Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Initially, applicants hereby admit that for purposes of preparing chimeric genes of the present invention, as well as expression systems, host cells, and transgenic plants, plant seeds, or scion or rootstock cultivars that contain the chimeric genes of the present invention, and for practicing the claimed methods which recite use of the chimeric gene, the use of any one DNA molecule encoding a hypersensitive response elicitor protein or polypeptide derived from a bacterial plant pathogen (where the protein or polypeptide belongs to the art-recognized class of proteins —known as "harpins"— that contain substantially no cysteine, are glycine rich, heat stable, hydrophilic, and capable of eliciting a hypersensitive response in non-host plants) would have been obvious over the use of any other DNA molecule encoding a hypersensitive response elicitor protein or polypeptide derived from a bacterial plant pathogen (which protein belongs to the same art-recognized class). In view of this admission, applicants submit that it is improper for claims 11-21 and 45-55 to remain withdrawn. Pending claims 1-73 should be examined in their entirety.

The rejection of claims 1-10, 22-44, and 56-73 under 35 U.S.C. § 112 (first paragraph) as lacking enablement is respectfully traversed.

The U.S. Patent and Trademark Office ("PTO") has taken the position that the above-identified application fails to enable the use of: (1) nucleic acid molecules encoding a hypersensitive response elicitor protein or polypeptide other than HrpN of *Erwinia amylovora* (SEQ ID NO: 4); (2) any oomycete-inducible promoter other than *gst1* of potato; and (3) any fragments of the *gst1* promoter of SEQ ID NO: 9. Applicants respectfully disagree.

With respect to the first issue identified above, applicants submit that due to fact that HrpN of *Erwinia amylovora* is a member of an art-recognized class of proteins, known as "harpins", that share a number of physical properties and share the ability to cause several distinct plant responses, results achieved with one member of this class would be expected by one of ordinary skill in the art to be achieved with other members of this same class. In support of applicants' position, applicants rely on the previously submitted Declaration of Zhong-Min Wei Under 37 C.F.R. § 1.132 ("Wei Declaration")(submitted with response dated July 30, 2003).

The Wei Declaration provides evidence that HrpN of *Erwinia amylovora* is conserved among *Erwinia* pathogen, including *Erwinia chrysanthemi*, *Erwinia carotovora*,

and *Erwinia stewartii* (Wei Declaration ¶¶ 6-9), and that HrpZ of *Pseudomonas syringae* is conserved among *Pseudomonas syringae* pathovars (Wei Declaration ¶¶ 6, 10).

The Wei Declaration further identifies a number of shared characteristics that distinguish the hypersensitive response elicitors known as "harpins" from other proteins of plant pathogens. Biochemically, these hypersensitive response elicitors have a number of common characteristics (Wei Declaration ¶ 15), which include containing substantially no cysteine, being glycine rich, heat stable, hydrophilic, lacking of an N-terminal signal sequence, and susceptible to proteolysis (*Id.*; *see also* He et al., "*Pseudomonas syringae* pv. *syringae* Harpin$_{Pss}$: A Protein That is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," *Cell* 73:1255-1266 (1993) ("He") at 1262 (copy attached to July 30, 2003 response as Exhibit A)). He is incorporated by reference in its entirety into the specification of the present application (*see* page 15, lines 20-24; page 40, lines 1-3). In addition, hypersensitive response elicitors share a unique secondary structure that has been associated with these elicitors' distinct biological activities (Wei Declaration ¶ 16). The structure has two primary components, an alpha helix unit and a relaxed acidic unit having a sheet or random turn structure (*Id.*). In the absence of one or both of these components, hypersensitive response elicitation does not occur (*Id.*).

In addition to eliciting the hypersensitive response in a broad range of plant species, hypersensitive response elicitors that can be used in the present invention also share the ability to induce specific plant responses (Wei Declaration ¶ 17). The induction of plant disease resistance (Wei Declaration ¶¶ 18, 19, 22, 23), plant growth enhancement (Wei Declaration ¶¶ 20, 25, 26), and plant stress resistance (Wei Declaration ¶¶ 21, 29-33) are three plant responses that result from treatment of plants or plant seeds with a hypersensitive response elicitor from a bacterial plant pathogen.

The nucleotide sequences for several hypersensitive response elicitor proteins have been previously reported in the literature (*see* Wei Declaration ¶¶ 7-11 and Exhibits thereto) and several are disclosed in the above-identified application as SEQ ID NOs: 2 (encoding harpin$_{Ech}$ or HrpN$_{Ech}$), 4 (encoding harpin$_{Ea}$ or HrpN$_{Ea}$), 6 (encoding harpin$_{Pss}$ or HrpZ$_{Pss}$), and 8 (encoding PopA1). One of ordinary skill in the art is fully able to prepare chimeric genes using these and other known members of the art-recognized class of hypersensitive response elicitors using known recombinant techniques, such as those described in the specification (paragraph bridging pages 22-23 and Example 1). As demonstrated in the above-identified application, applicants provide experimental evidence at Example 3 (pages 37-39) showing that chimeric constructs formed with an oomycete-inducible promoter and a DNA molecule encoding HrpN, a member of the above-identified

art-recognized class of hypersensitive response elicitors, can impart oomycete resistance to transgenic plants that contain the chimeric gene. Because one of skill in the art can reasonably expect the results achieved using HrpN-encoding chimeric genes to be similarly achieved with chimeric genes encoding other members of the art-recognized class of hypersensitive response elicitor proteins, one of ordinary skill in the art would be fully able to prepare such chimeric genes and transgenic plants, with the plants being expected to express the chimeric gene to induce resistance against oomycetes.

As further evidence that expression of a hypersensitive response elicitor other than HrpN of *Erwinia amylovora* will be effective when expressed under control of a oomycete-inducible promoter, applicants would again like to bring Belbahri et al., "A Local Accumulation of the *Ralstonia solanacearum* PopA Protein in Transgenic Tobacco Renders a Compatible Plant-Pathogen Interaction Incompatible," *Plant J.* 28(4):419-430 (2001) ("Belbahri") (copy attached to July 30, 2003 submission as Exhibit E) to the attention of the PTO. Belbahri separately confirmed the breadth of the presently claimed invention by using a chimeric gene that contained the *hsr203J* promoter and a nucleic acid encoding PopA (e.g., fragment of SEQ ID NO: 8 of the present application) to induce oomycete resistance in transgenic tobacco plants that express the chimeric gene following oomycete infiltration. The PTO had previously refused to consider Belbahri because the reference was published after the filing date of the present application (office action at page 4). The PTO's failure to consider Belbahri is improper, because applicants have cited Belbahri not to supplement the disclosure of the present application but instead as evidence that the present application fully enables practice of the claimed invention. The Federal Circuit has determined that post-filing date disclosures can be relied upon as evidence that the claimed subject matter can be practiced according to the disclosure in the specification. *Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ2d 1302, 1305 (Fed. Cir. 1987). Belbahri does just that.

Because applicants have demonstrated that HrpN of *Erwinia amylovora* can be effective, under control of an oomycete-inducible promoter, to protect plants against oomycete-caused disease, and Belbahri confirmed that PopA is likewise useful in this regard, applicants submit that the present application would allow others fully to practice the presently claimed invention. This conclusion is consistent with applicants' demonstration that the hypersensitive response elicitor proteins or polypeptides that can be used in the present invention are members of an art-recognized class that are characterized by shared physical and biochemical properties, and behave similarly in their ability to induce specific plant responses.

The PTO suggests at page 4 of the outstanding office action that the specification fails to teach "*E. amylovora* hypersensitive response elicitor genes other than SEQ ID NO: 3." As noted above, the present application does teach three other hypersensitive response elicitor genes and, given confirmation by Belbahri that persons of skill in the art can practice the claimed invention without undue experimentation, applicants submit that the disclosure and working examples satisfy the enablement requirement with regard to the hypersensitive response elicitor genes belonging to the art-recognized class.

The PTO also suggests at page 4 that the specification fails to teach "hypersensitive response elicitor genes not yet isolated." The failure to identify in the specification every possible variation of the invention (including those that had not been identified prior to the filing date) has been held previously by the Federal Circuit to be of no import to the enablement inquiry. *U.S. Steel Corp. v. Phillips Petroleum Co.*, 865 F.2d 1247, 1250, 9 USPQ2d 1461, 1463 (Fed. Cir. 1989) ("A patent applicant is not required ... to predict every possible variation, improvement, or commercial embodiment of his invention.") Hence, the mere fact that other hypersensitive response elicitor proteins within the art-recognized class (and their encoding nucleic acids) have been identified does not preclude a determination that the present application fully enables those of skill in the art to practice the claimed invention with any member of the art-recognized class.

For the foregoing reasons, one of ordinary skill in the art would expect results achieved with one hypersensitive response elicitor of the art-recognized class to be similarly achieved with all other members of the art-recognized class.

With respect to the second issue identified above, applicants submit that the recitation of the requirements of the oomycete-inducible promoter fully enables one of ordinary skill in the art to practice the present invention with *gst1* (*prp1*-1) promoter as well as any other oomycete-inducible promoters. The criteria for selecting promoters useful in accordance with the present invention is recited at page 21, lines 22-24 of the above-identified application: "The promoter of the chimeric gene should be selected on the basis of its ability to induce transcription of the first DNA molecule in response to infection of the plant by an oomycete (i.e., the oomycete activates the promoter)." Applicants submit that one of ordinary skill in the art is fully capable of identifying whether or not a given promoter is oomycete-inducible. The procedures used for determining oocymete inducibility were known in the art prior to the filing date of the present application and, therefore, need not be disclosed for purposes of enabling one of ordinary skill in the art. For example, Keller et al., "Pathogen-Induced Elicitin Production in Transgenic Tobacco Generates a Hypersensitive Response and Nonspecific Disease Resistance," *Plant Cell* 11:223-235 (1999) ("Keller")

(already of record) reports on the use of the *hsr203J* promoter to express cryptogein in response to *Phytophthora parasitica* var. *nicotianae*. As noted above, Belbahri confirmed the breadth of the presently claimed invention by using a chimeric gene that contained the *hsr203J* promoter and a nucleic acid encoding PopA (e.g., fragment of SEQ ID NO: 8 of the present application) to induce oomycete resistance in transgenic tobacco plants expressing the chimeric gene following oomycete infiltration.

Because other oomycete-inducible promoters would be expected (and indeed subsequent to applicants' invention have been demonstrated) to be effective when preparing a chimeric gene of the present invention, one of skill in the art would be fully able to select previously known or hereafter developed oomycete-inducible promoters and use such promoters in accordance with the presently claimed invention. Therefore, this basis of rejection is improper.

With respect to the third issue identified above, applicants submit that the above amendments obviate the basis of this rejection. Specifically, claims 6 and 34 recite the minimal *gst1* promoter identified in the specification, nt 295-567 of SEQ ID NO: 9. Longer fragments of SEQ ID NO: 9 and the full-length sequence are also encompassed by the claim language. Therefore, this basis of rejection should be withdrawn.

For all of the above-noted reasons, the present application enables one of skill in the art to practice the presently claimed invention. Therefore, the rejection of claims 1-10, 22-44, and 56-73 under 35 U.S.C. § 112 (first paragraph) as lacking enablement should be withdrawn.

The rejection of claims 1-10, 22-44, and 56-73 under 35 U.S.C. § 112 (first paragraph) as lacking written descriptive support is respectfully traversed.

There appear to be two bases of rejection. First, the PTO suggests at page 5 of the outstanding office action that the "...specification does not describe the sequence of any nucleic acids encoding hypersensitive response elicitor proteins from *E. amylovora* other than SEQ ID NO: 4 nor does it describe the features that distinguish *E. amylovora* hypersensitive response elicitor genes from other hypersensitive response elicitor genes." Second, the PTO suggests at page 5 of the outstanding office action that the specification does not describe effective fragments of *gst1* or more than one *gst1* promoter. Applicants respectfully disagree.

With respect to the first basis of rejection, applicants submit that the PTO must consider, for purposes of the written description requirement, all species disclosed in the present application, not just the HrpN encoding nucleic acid of *Erwinia amylovora*. As noted above, the present application identifies by sequence, four nucleic acid molecules encoding hypersensitive response elicitor proteins derived from various bacterial plant pathogens: one

encoding HrpN of *Erwinia chrysanthemi* or harpin$_{Ech}$, one encoding HrpN of *Erwinia amylovora* or harpin$_{Ea}$, one encoding HrpZ of *Pseudomonas syringae* or harpin$_{Pss}$, and one encoding PopA1 of *Pseudomonas* (now *Ralstonia) solanacearum.*

As demonstrated in the Wei Declaration, harpin$_{Ea}$ is a representative species of the presently recited genus and results achieved with one member of the art-recognized class have proven to be similarly achieved with other members of the art-recognized class. Thus, one of skill in the art would appreciate that the results of Example 3 in the present application, using a chimeric gene encoding harpin$_{Ea}$, fully demonstrate that applicants were in possession of the presently claimed invention using the genus of hypersensitive response elicitor proteins derived from various bacterial plant pathogens.

That the present application supports the claim language "a hypersensitive response elicitor protein or polypeptide derived from a bacterial plant pathogen, which protein or polypeptide is characterized by containing substantially no cysteine, being glycine rich, heat stable, hydrophilic, and capable of eliciting a hypersensitive response in non-host plants" is entirely consistent with the Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶1, "Written Description" Requirement, 66 Fed. Reg. 1099 (January 5, 2001) ("Written Description Guidelines"), because the present application describes "a representative number of species."

The burden of establishing that an application lacks adequate written descriptive support falls on the PTO. *See In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) ("[T]he PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims."). In this case, the only assertion made by the PTO to support its conclusion is the absence of nucleotide sequences of other *Erwinia amylovora* hypersensitive response elicitors. Thus, the PTO cites no directly relevant evidence that the four disclosed species are not representative of the recited genus. However, the present application presents *direct* evidence of the structural and functional features shared by the four exemplary members of the recited genus (which, applicant submits, would allow one of ordinary skill in the art reasonably to conclude that applicant was in possession of the recited genus). Despite the overwhelming evidence that the four disclosed species are representative of the presently claimed genus, the PTO concludes that this is insufficient.

Nowhere has the PTO presented evidence that the four disclosed species of hypersensitive response elicitors are *not* representative of the recited genus, as required by *In re Wertheim*. The PTO has failed even to consider the fact that four species are disclosed. The Written Description Guidelines indicate that when the genus represents widely variant

species more than one species is required, yet when the genus represents closely related species as few as one species may be sufficient. 66 Fed. Reg. at 1106. Thus, size of the genus is clearly of less import than variance of species within the genus. In this case, the *direct* evidence presented in the specification demonstrates that a number of structural features are shared (the substantially low or non-existent cysteine content, high glycine content, heat stable, hydrophilic) and functional variance is at a minimum (capable of eliciting a hypersensitive response in non-host plants) (*see* application pages 9-18; He at 1262 (copy attached to July 30, 2003 response as Exhibit A)). The PTO, in sharp contrast to applicants, has provided no evidence concerning variance within the genus. Moreover, the PTO's position is inconsistent with the Written Description Guidelines, which clearly recites: "Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces." 66 Fed. Reg. at 1106. Because the four disclosed species are representative of the genus, applicants submit that this ground of rejection should be withdrawn.

   With respect to the second basis of rejection, applicants submit that the present application both identifies several effective fragments of the *gst1* promoter as well as identifies the property that would allow one of ordinary skill in the art to select other promoters useful in accordance with the present invention. While structural variance may exist for different oomycete-inducible promoters, the important criteria for selecting such promoters is not their structure *per se*, but instead their conserved functional property of being inducible upon infection of a plant by an oocmycete. Thus, other subsequently identified oomycete-inducible promoters could simply be substituted for the *gst1* promoter as used in the present application (or the *hsr203J* promoter used by Belbahri and Keller). Therefore, one of ordinary skill in the art would appreciate that the results of Example 3 in the present application, using a chimeric gene formed with the *gst1* promoter, fully demonstrates that applicants were in possession of the presently claimed invention using the genus of oomycete-inducible promoters.

   For all of the above-noted reasons, the present application does satisfy the written description requirement given that the application does describe a number of species that can be used in accordance with the presently claimed genus and, the results achieved using the *gst1:harpin$_{Ea}$* chimeric gene are predictive of other species within the claimed genus. The PTO has failed to provide any evidence to the contrary.

   Therefore, the rejection of claims 1-10, 22-44, and 56-73 under 35 U.S.C. § 112 (first paragraph) as lacking written descriptive support is improper and should be withdrawn.

The rejection of claims 1-10, 22-44, and 56-73 under 35 U.S.C. § 112 (second paragraph) is respectfully traversed in view of the above amendments and the following remarks.

The use of relative terminology in claim language does not automatically render a claim indefinite. *Seattle Box Co. v. Indus. Crating & Packing, Inc.*, 731 F.2d 818, 826, 221 USPQ 568, 573-74 (Fed. Cir. 1984). The crucial factor in assessing the definiteness or indefiniteness of relative language is whether one of ordinary skill in the art — in view of the prior art and status in the art — would be apprised of the scope of the invention. *See Manual of Patent Examining Procedure* § 2173.05 (2003). Applicants have already demonstrated, via the Wei Declaration, that the bacterial plant pathogen-derived hypersensitive response elicitor proteins recited in the claim language are an art-recognized class. Further evidence that the recited properties, including the property of being 'glycine rich', are shared by this class of proteins is afforded by Büttner et al., "Getting Across — Bacterial Type III Effector Proteins on Their Way to the Plant Cell," *EMBO Journal* 21(20):5313-5322 (2002) ("Büttner") (copy attached as hereto as Exhibit 1) at 5318, left column; and Alfano et al., "Minireview: The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," *J. Bacteriol.* 179(18):5655-5662 (1997) ("Alfano") (copy attached hereto as Exhibit 2) at 5657, right column.

From the foregoing, it should be appreciated that persons of skill in the art regularly use the term "glycine-rich" to define the presently recited, art-recognized class of bacterial hypersensitive response elicitor proteins. Thus, although the term does not have an exact numerical meaning, it is recognized by persons of skill in the art as having a sufficiently precise meaning.

For these reasons, the rejection of claims 1-10, 22-44, and 56-73 under 35 U.S.C. § 112 (second paragraph) for indefiniteness is improper and should be withdrawn.

The rejection of claims 1-2, 6-7, 22-28, 30-31, 34-36, 39-41, 56-60, 62, 64-65, 68-70, and 72-73 under 35 U.S.C. § 102(a) as being anticipated by Keller is improper. As evidenced by the accompanying Declaration of Steven V. Beer and David W. Bauer Under 37 C.F.R. § 1.131 ("Rule 131 Declaration") and Exhibit A attached thereto, the presently claimed invention was reduced to practice by Drs. Bauer and Beer in the United States prior to February 1999. For this reason, Keller is not available as prior art. Therefore, the rejection is improper and should be withdrawn.

The rejection of claims 1-2, 5-10, 22-31, 34-36, 41-44, 56-60, 62-65, 70, and 73 under 35 U.S.C. § 102(a) as being anticipated by Abdul-Kader et al., "Evaluation of the *hrpN* Gene for Increasing Resistance to Fire Blight in Transgenic Apple," *Proc. of the 8th Int. Workshop on Fire Blight*, Momol and Saygili, eds., *Acta Horticulture* 489:247-250 (1999) ("Abdul-Kader") is improper. As evidenced by the accompanying Rule 131 Declaration and Exhibit A attached thereto, the presently claimed invention was reduced to practice by Drs. Bauer and Beer in the United States prior to February 1999. For this reason, Abdul-Kader is not available as prior art. Therefore, the rejection is improper and should be withdrawn.

The rejection of claims 1-10, 22-36, 41-44, 56-65, 70-71, and 73 under 35 U.S.C. § 103(a) for obviousness over Abdul-Kader in view of Pfitzner et al., "Isolation and Characterization of cDNA Clones Encoding Pathogenesis-related Proteins from Tobacco Mosaic Virus Infected Tobacco Plants," *Nucl. Acids Res.* 15:4449-4465 (1987) ("Pfitzner") is respectfully traversed. For the reasons noted above, Abdul-Kader is not available as prior art. The PTO cites to Pfitzner only for the teaching of various pathogenesis-related proteins and their secretion signals. Because Pfitzner alone fails to teach or suggest each and every limitation of the claimed invention, Pfitzner cannot have rendered obvious the invention defined by claims 1-10, 22-36, 41-44, 56-65, 70-71, and 73. Therefore, this rejection is improper and should be withdrawn.

The rejection of claims 1-2, 5-10, 22-31, 34-38, 41-44, 56-67, 70-71, and 73 under 35 U.S.C. § 103(a) for obviousness over Abdul-Kader in view of Scorza et al., "Producing Transgenic 'Thompson Seedless' Grape (*Vitis vinifera* L.) Plants," *J. Amer. Soc. Hort. Sci.* 121:616-619 (1996) ("Scorza") is respectfully traversed. For the reasons noted above, Abdul-Kader is not available as prior art. The PTO cites to Scorza only for the teaching of *Agrobacterium* and biolistic transformation of grape plant cells. Because Scorza alone fails to teach or suggest each and every limitation of the claimed invention, Scorza cannot have rendered obvious the invention defined by claims 1-2, 5-10, 22-31, 34-38, 41-44, 56-67, 70-71, and 73. Therefore, this rejection is improper and should be withdrawn.

The rejection of claims 1-4, 6-7, 22-28, 30-33, 34-36, 39-41, 56-62, 64-65, and 68-73 under 35 U.S.C. § 103(a) for obviousness over Keller in view of Pfitzner is respectfully traversed. For the reasons noted above, Keller is not available as prior art. Because Pfitzner alone fails to teach or suggest each and every limitation of the claimed invention, Pfitzner cannot have rendered obvious the invention defined by claims 1-4, 6-7, 22-28, 30-33, 34-36,

39-41, 56-62, 64-65, and 68-73. Therefore, this rejection is improper and should be withdrawn.

The rejection of claims 1-2, 6-10, 22-28, 30-31, 34-44, 56-62, and 64-73 under 35 U.S.C. § 103(a) for obviousness over each of Chappell and Keller in view of U.S. Patent No. 5,977,060 to Zitter et al. ("Zitter") is respectfully traversed.

For the reasons noted above, Keller is not available as prior art against the presently claimed invention. Therefore, only the rejection of the above-noted claims over Chappell in view of Zitter is addressed below.

Chappell teaches the preparation of a chimeric gene including (1) an inducible transcription regulatory sequence of the EAS4 gene (-167 to -100 nt) (col. 9, lines 36-63); (2) a promoter containing either the EAS4 promoter (col. 9, line 64 to col. 10, line 3) or a cauliflower mosaic virus 35S minimal promoter (col. 8, line 66 to col. 9, line 35); (3) a DNA molecule encoding an elicitin, a chitinase, TMV coat protein or other plant virus coat proteins, NIa virus gene, etc. (col. 10, lines 14-39 and col. 5, line 29 to col. 6, line 9); and (4) a transcription termination sequence (col. 6, lines 63-65). Chappell defines elicitins as including proteins produced by fungal plant pathogens, which proteins elicit a hypersensitive response in an infected plant (col. 5, lines 29-31) and indicates that certain bacterial proteins that also cause a hypersensitive response, such as AvrBs3 and AvrD avirulence proteins, can be considered elicitins (col. 5, line 61 to col. 6, line 9).

The PTO cites to Zitter only for teaching the use of *Erwinia amylovora hrpN* in transgenic plants and plant seeds, including grape plants.

The fungal "elicitins" are known *not* to resemble any other class of proteins (Ponchet et al., Review: "Are Elicitins Cryptograms in Plant-Oomycete Communications?" *CMLS* 56:1020-1047 (1999)("Ponchet")(copy attached hereto as Exhibit 3) at 1021, right column. The elicitins are generally characterized by their source (only expressed by *Phytophthora* and a few *Pythium*) (*id.*), their size (usually 98 amino acids) (*id.*), the occurrence of six conserved cysteine residues (responsible for formation of three disulfide bridges) (*id.*), and are synthesized as preproteins that are post-translationally modified by removal of an 18-20 amino acid signal peptide (*id.* at 1025, left column).

The avirulence (Avr) proteins are effector proteins expressed by bacterial plant pathogen (Collmer et al., "Genomic Mining Type III Secretion System Effectors in *Pseudomonas syringae* Yields New Picks for All TTSS Prospectors," *Trends in Microbiology* 10(10):462-469 ("Collmer") (copy attached to July 30, 2003 response as Exhibit D) at 463, Box 1; Büttner at 5318, left column). Avr proteins trigger an R-gene-specific plant defense reaction which often results in a hypersensitive response (Büttner at 5318, left column). The

activity of Avr proteins is most likely the result of translocation into plant cells (*id.*; Alfano at 5659). This has since been demonstrated for AvrBs3 of *Xanthomonas campestris* pv. *vesicatoria*, which localizes to the plant cell nucleus (Büttner at 5318-5319).

The "harpin" proteins of bacterial plant pathogens are, as noted above, glycine rich, substantially free of cysteine, heat stable, hydrophilic, and capable of eliciting a hypersensitive response in non-host plants (see pages 9-18 of the present application; He at 1262 (copy attached to July 30, 2003 response as Exhibit A); Büttner at 5318; Collmer at 463, Box 1 (copy attached to July 30, 2003 response as Exhibit D)). Unlike effector proteins, such as the Avr proteins, "harpin" proteins are believed to function as helper proteins within the plant apoplast (Collmer at 463, Box 1 (copy attached to July 30, 2003 response as Exhibit D)).

Because the Chappell definition of the term 'elicitin' is contrary to the commonly-accepted definition of the term (noted above), one of ordinary skill in the art is left to speculate which bacterial proteins are considered by Chappell as having similar effects on the hypersensitivity response as those of *P. parasitica* ParA1 elicitin. As examples, Chappel identifies two such bacterial proteins, the Avr proteins AvrBs3 (e.g., of *Xanthomonas campestris* pv. *vesicatoria*) and AvrD (e.g., of *Pseudomonas syringae* pv. *tomato*) (*see* Chappel at column 5, line 61 to column 6, line 9). Neither AvrBs3 nor AvrD is a member of the presently claimed class of hypersensitive response elicitor proteins. Thus, one of ordinary skill in the art would not construe the Chappell definition of "elicitin" to encompass use of the presently claimed class of hypersensitive response elicitor proteins or polypeptides. Nor would one of ordinary skill in the art believe Chappell to have suggested replacing the elicitin-encoding DNA molecule with a DNA molecule as presently claimed. Notably, Chappell does specify a use for "harpin" protein, but that use is for inducing expression of the EAS4-derived inducible transcriptional regulatory sequences (*see* Chappel at col. 9, lines 54-57) rather than as the expressed protein itself (as presently claimed).

The combination of Chappell and Zitter fail to teach or suggest the presently claimed invention. In particular, Chappell's teaching of the use of "harpin" proteins only for inducing expression of the encoded "elicitins" would suggest to one or ordinary skill in the art that the "harpin" proteins (e.g., including $HrpN_{Ea}$ as taught by Zitter) should not be used in the inducible chimeric gene of Chappell. The PTO has failed to address this inconsistency of Chappell, because the language appearing in the background of the invention (cited by the PTO) is inconsistent with the commonly accepted definition of "elicitin" and also inconsistent with the subsequent definition of elicitin that appears under the description of the invention (noted above).

Because Chappell is deficient for the reasons noted above, and Zitter fails to overcome the deficiencies of Chappell, the presently claimed invention would not have been obvious over Chappell in view Zitter. Therefore, the rejection of claims 1-2, 6-10, 22-28, 30-31, 34-44, 56-62, and 64-73 should be withdrawn.

In view of all the foregoing, it is submitted that this case is in condition for allowance and such allowance is earnestly solicited.
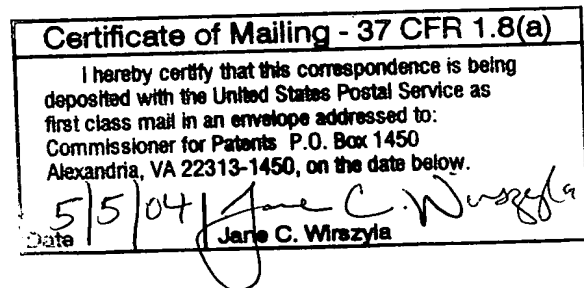
Respectfully submitted,

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NEW EMBO MEMBER'S REVIEW

Getting across—bacterial type III effector proteins on their way to the plant cell

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Pathogenicity of most Gram-negative bacterial plant pathogens depends on *hrp* (hypersensitive response and pathogenicity) genes, which control the ability to cause disease and to elicit specific defense responses in resistant plants. *hrp* genes encode a specialized type III secretion (TTS) system that mediates the vectorial delivery of bacterial effector proteins across both bacterial membranes as well as across the eukaryotic plasma membrane into the host cell cytosol. One well-studied effector protein is AvrBs3 from *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot in pepper and tomato. AvrBs3 induces hypertrophy symptoms in susceptible plants and triggers a resistance gene-specific cell death reaction in resistant plants. Intriguingly, AvrBs3 has characteristic features of eukaryotic transcription factors, suggesting that it modulates the host's transcriptome. Here, we discuss the TTS system of *X.campestris* pv. *vesicatoria* in the light of current knowledge on type III-dependent protein secretion in plant pathogenic bacteria.

Keywords: AvrBs3/*hrp* genes/pathogenicity island/PIP box/secretion

Introduction

Plants provide an attractive nutrient reservoir and ecological niche for bacterial pathogens. In most higher plants, bacterial colonization leads to a variety of severe diseases. However, disease is the exception rather than the rule since most plants possess a battery of defense mechanisms that repel invading microbes. Therefore, Gram-negative plant pathogenic bacteria have evolved sophisticated strategies to colonize their host plants. They enter the plant through natural openings such as stomata, or wounds, and multiply in the intercellular spaces of the tissue at the expense of the host.

Over the past two decades, genetic and molecular studies unraveled important mechanisms underlying bacterial pathogenicity. Essential for the molecular cross-talk between pathogens and their host plants is a specialized protein delivery system, the type III secretion (TTS) system. TTS systems are conserved in plant and animal pathogenic bacteria and mediate the vectorial delivery of bacterial effector proteins into the host cell (Hueck, 1998; Cornelis and Van Gijsegem, 2000). In plant pathogens, TTS systems are encoded by *hrp* (hypersensitive response

and pathogenicity) genes, essential determinants of bacterial pathogenicity that control the ability to multiply in susceptible hosts and to cause disease (Alfano and Collmer, 1997). In addition, *hrp* genes are required to induce the hypersensitive response (HR), a rapid localized programmed death of plant cells at the infection site, in resistant host and in non-host plants (Klement, 1982). The HR is part of the plant's innate immune response that halts bacterial ingress. Induction of the HR is due to the specific recognition of a bacterial effector protein [designated avirulence (Avr) protein] by a corresponding plant resistance (R) protein (Flor, 1971; Table I).

Among the model organisms for the molecular and genetic characterization of host–plant interactions and the functional analysis of TTS systems are *Erwinia amylovora*, *Ralstonia solanacearum*, pathovars (pv.) of *Pseudomonas syringae* and species (spp.) of *Xanthomonas*, all infecting important crop plants. The pathovar designation refers to differences in the host range of the bacteria. For some of these bacteria, the genome sequence has become available recently, initiating a new era in molecular plant pathology (Da Silva *et al.*, 2002; Salanoubat *et al.*, 2002; www.tigr.org). Our laboratory studies *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot in pepper and tomato plants, which is the focus of this review.

The *hrp* pathogenicity island—genetic requisite for effector protein traffic

Gram-negative bacteria utilize different protein secretion systems to transport proteins across the inner and outer membrane. Among the six main groups of secretion systems, TTS systems exhibit the most complex architecture (Thanassi and Hultgren, 2000). Around 20 proteins are involved in the formation of a membrane-spanning secretion apparatus, which is associated with an extracellular filamentous structure (Hueck, 1998; see below).

Type III-mediated protein secretion into the extracellular medium was discovered initially in the animal pathogen *Yersinia enterocolitica* (Heesemann *et al.*, 1984). However, the first genes encoding components of the TTS system were identified by the analysis of non-pathogenic mutants of the plant pathogens *P.syringae* pv. *syringae* and *P.syringae* pv. *phaseolicola* (Niepold *et al.*, 1985; Lindgren *et al.*, 1986). Except for *Agrobacterium* spp., *hrp* genes are present in all Gram-negative biotrophic plant pathogens and are generally organized in large clusters comprising >20 genes (Boucher *et al.*, 1987; Steinberger and Beer, 1988; Barny *et al.*, 1990; Arlat *et al.*, 1991; Bonas *et al.*, 1991). Based on similarities in *hrp* gene organization and regulation, plant pathogenic bacteria have been classified into two groups, group I (*E.amylovora* and *P.syringae*) and group II (*R.solanacearum* and species

of *Xanthomonas*) (Alfano and Collmer, 1996). At least nine *hrp* genes (termed *hrc* for *hrp* conserved) are conserved in both groups and encode components of the TTS system, which are also present in animal pathogenic bacteria (Bogdanove *et al.*, 1996; He, 1998; Hueck, 1998). Hrc proteins presumably constitute the core components of the secretion apparatus in the inner and outer membrane. With the exception of HrcC—the best studied Hrc protein, which belongs to the secretin family of outer membrane proteins—Hrc proteins share sequence similarities with flagellar assembly components. The flagellar assembly apparatus serves as a protein export system and probably represents an evolutionary ancestor of the TTS system (Hueck, 1998; Macnab, 1999; Aizawa, 2001; Young and Young, 2002).

In contrast to conserved *hrp* genes, the precise role of non-conserved *hrp* genes remains to be investigated. Genetic studies of *X.campestris* pv. *vesicatoria* revealed that type III secretion requires at least six non-conserved *hrp* genes, some of which encode type III-secreted

proteins, e.g. HrpB2 (Rossier *et al.*, 2000; Table II). The *hrp* region also contains so-called *hrp*-associated (*hpa*) genes (Figure 1), which are not essential for bacterial pathogenicity but contribute to the interaction with the host plant (Huguet *et al.*, 1998; Noël *et al.*, 2002; O.Rossier, D.Büttner and U.Bonas, unpublished data).

How did *hrp* gene clusters evolve? Genes involved in bacterial virulence often are located in regions that show characteristics of pathogenicity islands. These DNA regions usually are flanked by direct repeats, insertion sequence (IS) elements, tRNA genes and/or genes for integrases and transposases. Pathogenicity islands often differ in G + C content from the genomic DNA, indicating horizontal gene transfer (Hacker and Kaper, 2000). In *X.campestris* pv. *vesicatoria*, mobility of the *hrp* region has indeed been observed (Basim *et al.*, 1999). Furthermore, sequence analyses of DNA regions flanking the *hrp* gene cluster revealed the presence of an IS-like element and putative effector genes with lower G + C content than the genomic DNA (Noël *et al.*, 2002; Figure 1).

Typical features of pathogenicity islands are also present in DNA sequences flanking the *hrp* gene cluster of *P.syringae*. Here, the region adjacent to *hrpK* has a lower G + C content and contains sequences homologous to IS elements, transposases and tRNA genes. Interestingly, the genes located in this region, termed exchangeable effector locus (EEL), vary in pathovars of *P.syringae* (Alfano *et al.*, 2000; one example is given in Figure 1).

Entering the plant—green light for *hrp* gene expression

Type III secretion is a regulated process. Genes encoding components of the secretion apparatus are not constitutively expressed but activated *in planta* and in minimal media mimicking the environmental conditions present in the plant apoplast (Lindgren, 1997). Proteins involved in

Table I. R gene-specified pathogen recognition according to gene-for-gene interactions[a]

Pathogen genotype	Plant reaction	
	Host plant genotype[b] R1/R1 or R1/r1	r1/r1
avr1	HR[d]	Disease
_[c]	Disease	Disease

[a]Gene-for-gene hypothesis (Flor, 1971).
[b]*R1*, resistance locus allowing recognition of a corresponding avirulence (*avr*) gene (designated *avr1*). Most resistance (*R*) genes are single dominant genes. *r1* refers to the absence of a functional *R1* allele.
[c]The avirulence gene is absent or mutated, resulting in loss of recognition by plants carrying the corresponding *R* gene.
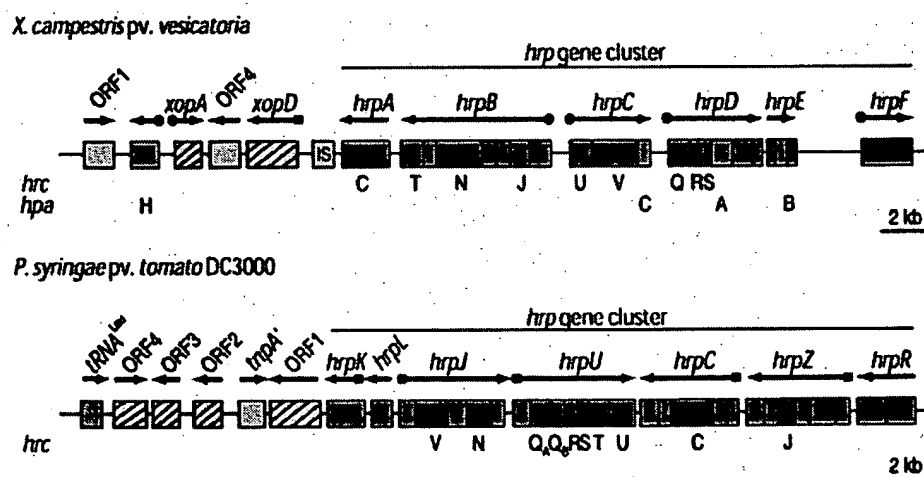[d]HR, hypersensitive reaction.

Fig. 1. Schematic overview of the *hrp* gene clusters and the left flanking regions from *X.campestris* pv. *vesicatoria* (group II) and *P.syringae* pv. *tomato* DC3000 (group I). The regions contain *hrp*, *hrc* and *hpa* genes (represented in blue, red and green, respectively). Arrows indicate the direction of transcription. Black dots and squares refer to the presence of PIP and *hrp* boxes, respectively. Hatched regions correspond to sequences with low G + C content; yellow regions refer to mobile genetic elements.

Table II. *Xanthomonas campestris* pv. *vesicatoria* type III-secreted proteins

Protein[a]	Characteristics/homology[b]	Expression[c]	References
Components of the TTS apparatus			
HrpB2[‡]	Extracellular component of the TTS system	Induced; PIP box	Wengelnik and Bonas (1996); Rossier *et al.* (2000)
HrpE1[‡]	Major Hrp pilus subunit	Basal expression, induction	Wengelnik and Bonas (1996); Rossier (1999); T.Ojanen-Reuhs and U.Bonas (unpublished data)
HrpF	Translocon protein	Induced; PIP box	Wengelnik and Bonas (1996); Rossier *et al.* (2000); Büttner *et al.* (2002)
Xops[d]			
√XopA	Hpa1 (*X.oryzae* pv. *oryzae*)	Induced; PIP box	Noël *et al.* (2002)
XopB	AvrPphD (*P.syringae* pv. *phaseolicola*)	Induced	Noël (2001); Noël *et al.* (2001)
XopC		Induced	Noël (2001); Noël *et al.* (2001)
XopD	PsvA (*P.syringae* pv. *eriobotryae*)	Induced; *hrp* box	Noël *et al.* (2002)
XopJ	AvrRxv/YopJ family; putative cysteine protease	Induced	Noël (2001); Noël *et al.* (2001)
√HpaA	NLS	Induced; PIP box	Wengelnik and Bonas (1996); Huguet *et al.* (1998)
AvrBs1*	AvrA (*P.syringae* pv. *glycinea*)	Constitutive	Ronald and Staskawicz, (1988); Escolar *et al.* (2001)
√AvrBs2*	Agrocinopine synthase (*A.tumefaciens*); phosphodiesterase (*E.coli*)	ND	Kearney and Staskawicz (1990); Swords *et al.* (1996); Mudgett *et al.* (2000)
√AvrBs3*	NLS; AAD; AvrBs3 family	Constitutive[e]	Van den Ackerveken *et al.* (1996); Rossier *et al.* (1999); Szurek *et al.* (2001); Marois *et al.* (2002)
AvrBs4*	NLS; AAD; AvrBs3 family	Constitutive[e]	Bonas *et al.* (1993); Ballvora *et al.* (2001)
AvrBsT*	AvrRxv/YopJ family; putative cysteine protease	Constitutive	Escolar *et al.* (2001)
AvrRxv	AvrRxv/YopJ family; putative cysteine protease	Constitutive; PIP box	Ciesiolka *et al.* (1999); Rossier *et al.* (1999)
AvrXv3*	AAD	Induced; PIP box	Astua-Monge *et al.* (2000a)
AvrXv4	AvrRxv/YopJ family; putative cysteine protease	ND; PIP box	Astua-Monge *et al.* (2000b)

[a]‡, essential for type III secretion *in vitro*; √, virulence activity demonstrated; *, indicates ability of Avr proteins to induce the HR upon transient expression in resistant plants.
[b]AAD, acidic activation domain; NLS, nuclear localization signal; Yop, *Yersinia* outer protein.
[c]Expression *in planta* or under *hrp* gene-inducing conditions. ND, not determined; PIP, plant-inducible promoter.
[d]Xops, *Xanthomonas* outer proteins, include type III-secreted proteins with unknown destination as well as avirulence (Avr) proteins; Hpa, *hrp* associated.
[e]Recent *in vitro* expression experiments indicate that *hrpG** leads to a 2- to 3-fold increase in expression (U.Bonas *et al.*, unpublished data).
√, virulence activity demonstrated.
*, indicates ability of Avr proteins to induce the HR upon transient expression in resistant plants.

hrp gene regulation vary in the different groups of plant pathogens. In *X.campestris* pv. *vesicatoria*, *hrp* gene expression is controlled by HrpX, an AraC-type transcriptional activator (Wengelnik and Bonas, 1996). In minimal medium or *in planta*, the expression of *hrpX* is activated by HrpG, a transcriptional activator of the OmpR family of two-component regulators (Wengelnik *et al.*, 1996; Figure 2A). Recent transcriptome analysis revealed that HrpG, in most cases via HrpX, controls a genome-wide regulon including *hrp* genes and genes encoding *Xanthomonas* outer proteins (Xops; Wengelnik and Bonas, 1996; Astua-Monge *et al.*, 2000a; Noël *et al.*, 2001, 2002).

Interestingly, one of the *xop* genes, *xopD*, contains an *hrp* box-like motif in the promoter region (Figure 1; Table II). The *hrp* box is a conserved consensus sequence which was identified in promoters of *hrp* and effector genes in *P.syringae*. It presumably provides the binding site for HrpL, a member of the extracytoplasmic function family of sigma factors (Innes *et al.*, 1993; Xiao and Hutcheson, 1994; Xiao *et al.*, 1994; Fouts *et al.*, 2002). In *X.campestris* pv. *vesicatoria*, however, expression of *xopD* is controlled by HrpG and HrpX (Noël *et al.*, 2002). *xopD* encodes a putative type III effector protein with homology to the virulence factor PsvA from *P.syringae* pv. *eriobotryae* (Noël *et al.*, 2002; Table II). The presence

of an *hrp* box in the *xopD* promoter and the low G + C content of *xopD* support the hypothesis that genes involved in bacterial virulence might have been acquired during evolution by horizontal gene transfer.

Many *hrpX*-regulated genes of *X.campestris* pv. *vesicatoria* contain a PIP (plant-inducible promoter, consensus TTCGC-N$_{15}$-TTCGC) box in their promoter regions. This sequence motif might be involved in the HrpX-mediated gene regulation (Fenselau and Bonas, 1995; Wengelnik and Bonas, 1996; Noël *et al.*, 2002). However, there are also *hrpX*-independent promoters that contain a PIP box, e.g. *avrRxv* (Table II), indicating that the PIP box is not sufficient to confer inducibility by HrpX. In addition, the promoters of several *xop* genes that are controlled by HrpG and HrpX do not contain PIP boxes (Table II). Thus, it remains speculative whether the PIP box serves as a control element. So far, direct binding of HrpX to PIP box-containing promoter sequences could not be demonstrated (L.Escolar and U.Bonas, unpublished data).

PIP box-like motifs have also been identified in *Xanthomonas axonopodis* pv. *citri* and *X.campestris* pv. *campestris* in the promoters of *hrp* genes as well as genes encoding putative proteins with type II signal peptides and sequence homologies to cell wall-degrading enzymes, proteases and an iron receptor (Da Silva *et al.*, 2002). Furthermore, PIP box-like promoter sequences have been
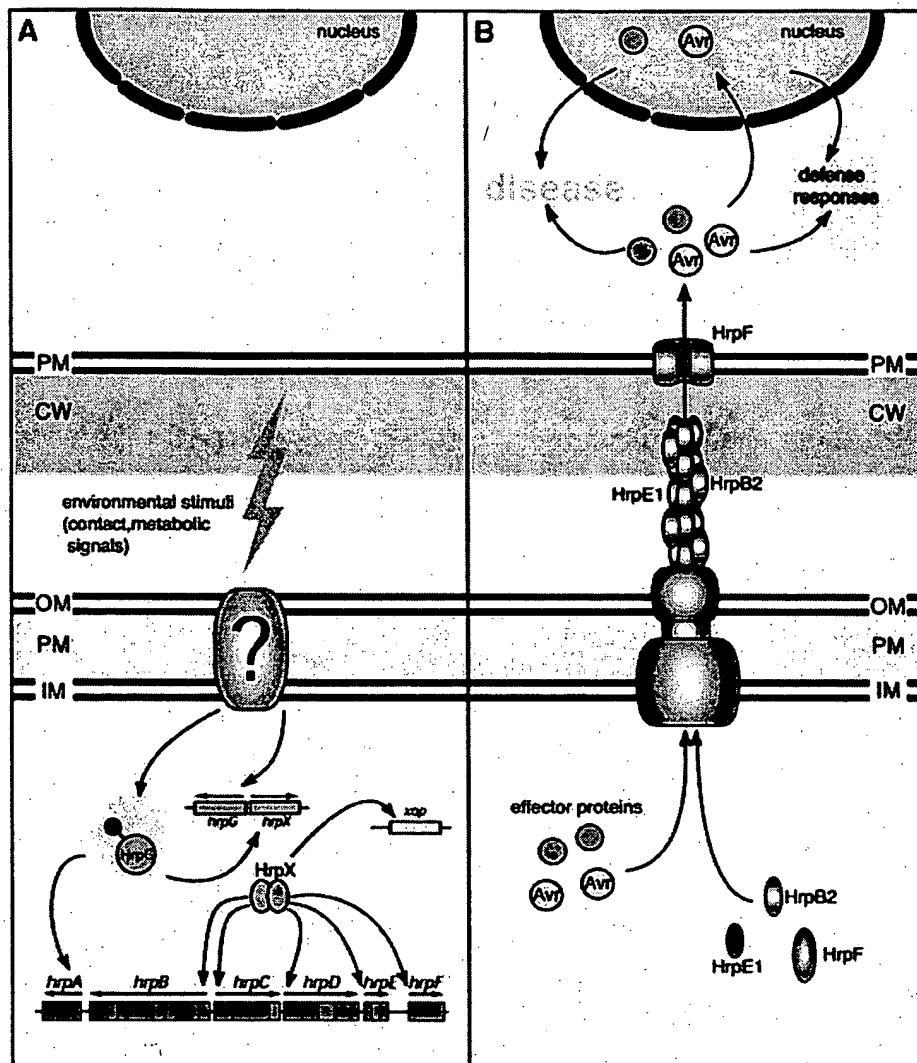
Fig. 2. Model for *hrp* gene regulation and type III secretion in *X.campestris* pv. *vesicatoria*. (A) A so far uncharacterized signal transduction system in the bacterial envelope (indicated by a question mark) senses environmental stimuli and transduces the signal to HrpG. HrpG activates the expression of *hrpA* and, via HrpX, the expression of *hrpB–hrpF* as well as of a number of *xop* genes. (B) Expression of *hrp* genes is essential for the formation of the TTS apparatus, which spans both bacterial membranes and mediates secretion of Hrp and effector proteins. The TTS apparatus is associated with the Hrp pilus, which presumably spans the cell wall (200 nm thick; not drawn to scale). The major subunit of the Hrp pilus is HrpE1. Translocation of effector proteins across the plant plasma membrane requires HrpF, the putative pore-forming component of the type III translocon. Effector proteins are targeted to different locations in the plant cell and presumably modulate cellular processes leading to disease symptom formation in susceptible plants. In resistant plants, effector proteins (designated Avr proteins) can be recognized and trigger the activation of specific defense responses. CW, cell wall; IM, inner membrane; OM, outer membrane; PM, plasma membrane.

identified in *R.solanacearum*, upstream of *hrp* transcription units, the *popA* gene and several *avr* gene homologs (Fenselau and Bonas, 1995; Wengelnik and Bonas, 1996; Salanoubat *et al.*, 2002). In *R.solanacearum*, *hrp* genes are controlled by HrpG and HrpB, which are homologous to HrpG and HrpX, respectively, from *X.campestris* pv. *vesicatoria* (Genin *et al.*, 1992; Brito *et al.*, 1999). In *R.solanacearum*, the outer membrane protein PrhA (plant regulator of *hrp* genes) presumably is on top of the regulatory cascade leading to *hrp* gene expression. PrhA is homologous to TonB-dependent siderophore receptors and acts as a sensor for a non-diffusible molecule present in the plant cell wall (Marenda *et al.*, 1998; Brito *et al.*, 1999,

2002; Aldon *et al.*, 2000). In contrast to *R.solanacearum*, the receptor(s) in *X.campestris* pv. *vesicatoria* that transmits external stimuli into the bacterial cell is still unknown (Figure 2).

Hrp pilus—tunnel to the host cell

TTS systems have been visualized in the animal pathogens *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*, and show striking morphological similarities to flagellar basal bodies: a membrane-embedded complex is associated with an extracellular hollow struc-

ture, the needle (Kubori et al., 1998; Tamano et al., 2000; Blocker et al., 2001; Sekiya et al., 2001).

Type III-dependent surface appendages have also been identified in plant pathogenic bacteria, i.e. P.syringae pv. tomato, E.amylovora, R.solanacearum and X.campestris pv. vesicatoria. These so-called Hrp pili have a similar diameter (6–8 nm), but are considerably longer than the needles of animal pathogens (Roine et al., 1997; Van Gijsegem et al., 2000; Hu et al., 2001; Jin et al., 2001; T.Ojanen-Reuhs and U.Bonas, unpublished data). Since Hrp pili can extend to a length of several micrometers, they have been proposed to cross the plant cell wall (Romantschuk et al., 2001; Figure 2). In R.solanacearum and P.syringae pv. tomato, the pilin, which is the major subunit of the Hrp pilus, is required for type III secretion in vitro (Van Gijsegem et al., 2000; Wei et al., 2000). Recent immunocytochemical analyses in E.amylovora and P.syringae pv. tomato elegantly demonstrated that Hrp pili serve as conduits for secreted proteins (Brown et al., 2001; Jin and He, 2001; Jin et al., 2001; Li et al., 2002). So far, there are no indications that Hrp pili also mediate bacterial contact with the host cell. In R.solanacearum, mutation of hrpY, the gene encoding the major pilus subunit, does not affect attachment of the bacteria to cultured plant cells (Van Gijsegem et al., 2000).

Getting in touch—the type III translocon

Translocation across the eukaryotic plasma membrane probably requires the presence of type III-secreted bacterial proteins that form the type III translocon, a channel-like complex in the host plasma membrane (Büttner and Bonas, 2002). Putative components of the translocon have been described mainly in animal pathogens whereas they have not been identified so far in most plant pathogenic bacteria. To our knowledge, HrpF from X.campestris pv. vesicatoria is the first known candidate for a type III translocon protein in bacterial plant pathogens. Mutant studies revealed that HrpF, which is secreted by the TTS system, is dispensable for type III secretion in vitro but essential for the interaction with the plant (Rossier et al., 2000; Büttner et al., 2002). hrpF mutants are not able to grow and cause disease in susceptible plants and to induce the HR in resistant plants. When tested in artificial lipid bilayer systems, HrpF induced pore formation, suggesting that it might be the channel-forming core component of the type III translocon (Büttner et al., 2002; Figure 2). Pore-forming activity has been demonstrated for the putative type III translocon proteins LcrV and PcrV from Yersinia pseudotuberculosis and Pseudomonas aeruginosa, respectively, which do not show any sequence similarity to HrpF (Holmström et al., 2001).

In animal pathogenic bacteria, observations of protein–protein interactions between putative translocon proteins suggest that the type III translocon is a heterogeneous protein complex. For instance, LcrV presumably interacts with YopB and YopD to build a functional translocon (Sarker et al., 1998). In X.campestris pv. vesicatoria, it remains to be investigated whether additional proteins besides HrpF are involved in the formation of the type III translocon. So far, studies to identify HrpF interaction partners failed since HrpF is a 'sticky' protein, making it

difficult to show interaction specificity (D.Büttner and U.Bonas, unpublished data).

Carte d'accès—recognition by the TTS system

The mechanisms that control type III secretion in planta are still unknown. In Erwinia spp., P.syringae and R.solanacearum, several type III-secreted proteins could be detected in the culture supernatant after incubation of the bacteria in hrp gene-inducing medium (e.g. Gaudriault et al., 1997; Mudgett and Staskawicz, 1999; Van Gijsegem et al., 2000).

In X.campestris pv. vesicatoria, the isolation of a point mutation in hrpG (E44K, designated hrpG*), which leads to constitutive expression of hrp genes, was key for the establishment of an in vitro secretion assay (Rossier et al., 1999; Wengelnik et al., 1999). However, expression of the hrp genes is not sufficient to trigger type III secretion. The identification of secreted proteins requires the incubation of hrpG* bacteria in acidic minimal medium, which probably mimicks the plant's apoplast. Interestingly, the X.campestris pv. vesicatoria TTS system also secretes heterologous proteins such as PopA from R.solanacearum, AvrB from P.syringae and YopE from Y.pseudotuberculosis, indicating that the secretion signal is conserved among plant and animal pathogenic bacteria (Rossier et al., 1999).

What is the nature of the secretion signal in proteins traveling the TTS systems? It has been proposed that the signal resides in the N terminus of the secreted proteins. In Yersinia spp., the first 11–17 amino acids of Yersinia outer proteins (Yops) are sufficient to drive the type III-dependent secretion of a reporter protein (Sory et al., 1995; Schesser et al., 1996; Lloyd et al., 2001b). Similarly, in X.campestris pv. vesicatoria, the first 28 amino acids of AvrBs2 contain a functional secretion signal (Mudgett et al., 2000). Type III-secreted proteins in both plant and animal pathogens do not share any sequence conservations in their N termini. However, comparative sequence analyses of multiple type III-secreted proteins of P.syringae pathovars revealed similarities in their N-terminal amino acid composition, including a high content of serine residues (on average 16–18%; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). In X.campestris pv. vesicatoria, the serine content within the first 25 amino acids of known TTS substrates varies between 8% (HrpB2) and 32% (HrpF). This is significantly higher than the serine content in the N termini of non-secreted components of the TTS system (betweeen 0%, as in HrcN, and 12%, as in HrcT).

Since frameshift mutations in the N-terminal coding sequence did not abolish type III secretion of a reporter protein in Y.enterocolitica, the secretion signal was also predicted to reside in the 5' region of the mRNA (Anderson and Schneewind, 1997). This hypothesis, which assumes a co-translational secretion, is, however, discussed controversially in the field. For instance, the Yersinia YopE and YopH proteins are expressed even in the absence of a functional TTS system. In addition, mutations in YopE resulting in an altered mRNA structure did not abolish its type III secretion (Lloyd et al., 2001b).

The situation is complicated further by the finding that several effector proteins from animal pathogens require specific chaperones for type III secretion and translocation (Bennett and Hughes, 2000; Lloyd et al., 2001a). Recently, TTS chaperones have also been identified in plant pathogenic bacteria. DspB from E.amylovora and ShcA from P.syringae are essential for the stability and/or secretion of the pathogenicity factor DspA and the effector protein HopPsyA, respectively (Gaudriault et al., 2002; van Dijk et al., 2002).

Quo vadis—type III-secreted proteins

Harpins

The first proteins known to be secreted by the TTS system of bacterial plant pathogens were the harpins; HrpZ from P.syringae and PopA from R.solanacearum (He et al., 1993; Arlat et al., 1994). Harpins are small, heat-stable, glycine-rich proteins that lack cysteines and elicit a necrosis-like reaction when infiltrated into non-host plants (Wei et al., 1992; He et al., 1993; Arlat et al., 1994; Alfano et al., 1996; Gaudriault et al., 1998). Interestingly, HrpZ from P.syringae was found to bind to the plant plasma membrane and to form ion-conducting pores in artificial lipid bilayers (Lee et al., 2001a,b). However, the role of harpins is not well understood. In most cases, a contribution to bacterial virulence could not be demonstrated. Only in E.amylovora, a mutation of the harpin gene hrpN results in the formation of reduced disease symptoms in susceptible plants (Wei et al., 1992; Barny, 1995).

Effector proteins

The best studied effector proteins are the products of avr genes, which were first identified genetically without knowing that they encode TTS substrates. Since the isolation of the first avr gene, avrA from P.syringae pv. glycinea (Staskawicz et al., 1984), >40 bacterial avr genes have been identified, mainly in species of Pseudomonas and Xanthomonas (Vivian and Arnold, 2000). As mentioned above, avr genes trigger an R gene-specific plant defense reaction which often culminates in the HR. The HR phenotype is easy to follow and has been instrumental in the dissection of both bacterial pathogenicity and specific defense reactions in the plant. In the absence of the corresponding R gene, no recognition occurs and the infection leads to disease. There is accumulating evidence that Avr proteins probably act as virulence factors, manipulating host cellular processes for the pathogen's benefit and thus contributing to bacterial fitness and/or symptom formation in susceptible plants (White et al., 2000). However, it should be emphasized that mutations in putative effector genes often do not affect bacterial virulence under laboratory conditions, indicating that they play a minor role or have redundant functions.

Until recently, type III-dependent delivery of bacterial effector proteins into the host cell has not been proven. Strong indirect evidence for translocation was provided by the fact that avr genes induced an R gene-specific HR when expressed inside the plant cell (Bonas and Van den Ackerveken, 1997; Cornelis and Van Gijsegem, 2000). Furthermore, several type III-secreted proteins from plant pathogens contain typical eukaryotic features, indicating an activity inside the host cell (White et al., 2000). For instance, the putative myristoylation motifs of several Avr proteins in pathovars of P.syringae suggest a localization to the plant plasma membrane, which has indeed been shown for AvrB and AvrRpm1 (Nimchuk et al., 2000). In these proteins, the myristoylation motifs are crucial for the avirulence function. Further support for the hypothesis of type III-dependent delivery of bacterial effector proteins into the plant cell was provided by the analysis of the effector protein AvrBs2 from X.campestris pv. vesicatoria, which was fused translationally to an adenylate cyclase reporter from Bordetella pertussis (Casper-Lindley et al., 2002). Recently, the direct detection of a bacterial effector protein in the plant cell has been reported: AvrBs3 from X.campestris pv. vesicatoria could be visualized in nuclei of infected plant cells, using an AvrBs3-specific antibody (Szurek et al., 2002; see below).

Arrival—AvrBs3 localizes to the plant cell nucleus

Characteristic eukaryotic protein motifs are also present in members of the AvrBs3 protein family in species of Xanthomonas (Gabriel, 1999; Lahaye and Bonas, 2001). AvrBs3-like proteins are highly homologous (90–97% amino acid sequence identity) and all contain C-terminal nuclear localization signals and an acidic activation domain, which are features of eukaryotic transcription factors (Yang and Gabriel, 1995; Van den Ackerveken et al., 1996; Zhu et al., 1998, 1999; Yang et al., 2000; Ballvora et al., 2001; Szurek et al., 2001). Differences between the family members are restricted mainly to the central protein region, which consists of 13.5–25.5 nearly perfect 34-amino-acid repeats (Lahaye and Bonas, 2001).

The AvrBs3 protein family is named after the first isolated member, AvrBs3 from X.campestris pv. vesicatoria (Bonas et al., 1989). AvrBs3 is one of the few Avr proteins for which a role in symptom formation could be demonstrated. In susceptible host plants, AvrBs3 induces hypertrophy, an enlargement of mesophyll cells (Marois et al., 2002). Since the induction of hypertrophy symptoms depends on functional nuclear localization signals and the acidic activation domain, we speculate that AvrBs3 acts as a transcription factor in the host cell nucleus. The nuclear localization signals probably provide the admission ticket for AvrBs3 to use the host's protein traffic road into the nucleus. Indeed, yeast two-hybrid studies and in vitro pull-down assays revealed that AvrBs3 interacts with pepper importin α which, together with importin β, mediates nuclear protein import (Görlich et al., 1995; Szurek et al., 2001; Figure 3). Immunocytological analyses demonstrated that the nuclear localization signals are essential for the targeting of AvrBs3 to nuclei of infected plant cells (Szurek et al., 2002).

The hypothesis that AvrBs3 acts as a transcription factor is supported by transcriptome analyses of infected susceptible pepper plants. cDNA-AFLP (cDNA-amplified fragment length polymorphism) studies unraveled AvrBs3-induced genes, designated upa (up-regulated by AvrBs3; Marois et al., 2002). Sequence analyses revealed that several upa genes show homologies to auxin-induced
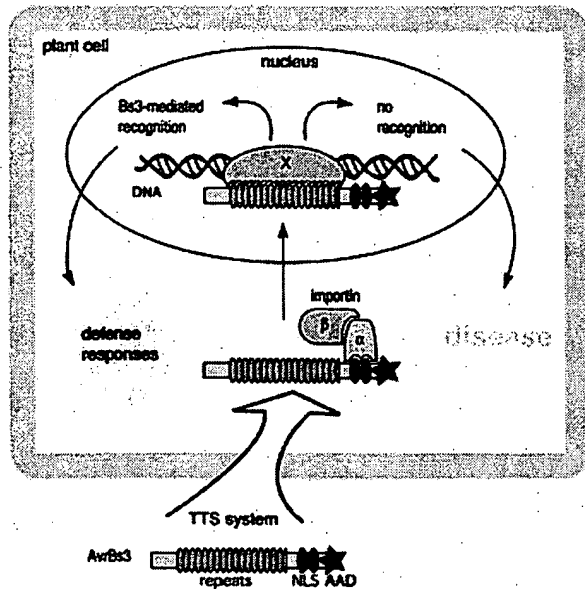
Fig. 3. Proposed model for the molecular mechanisms underlying virulence and avirulence activity of AvrBs3 from *X.campestris* pv. *vesicatoria*. Characteristic features of AvrBs3 are the central 17.5 nearly identical 34 amino acid repeats, two functional C-terminal nuclear localization signals (NLSs) and an acidic activation domain (AAD). Delivery of AvrBs3 into the host cell is mediated by the TTS system. In the plant cell, the NLSs bind to importin α, which together with importin β targets AvrBs3 to the plant cell nucleus. Direct or indirect (via a target protein X) interaction of AvrBs3 with the plant DNA leads to the modulation of the host's transcriptome and presumably results in hypertrophy, a disease symptom in susceptible plants. In resistant plants, specific plant defense responses are induced upon recognition of AvrBs3 by the R protein Bs3 (Bs, bacterial spot).

and expansin-like genes that usually play a role in cell enlargement.

Whether AvrBs3 induces gene expression with the aid of plant transcription factors or directly targets plant promoter sequences is not known (Figure 3). Support for a direct interaction of AvrBs3-like proteins with the host DNA comes from recent studies on AvrXa7, an AvrBs3 homolog from the rice pathogen *Xanthomonas oryzae* pv. *oryzae*, which directly binds to AT-rich DNA sequences (Yang *et al.*, 2000).

Perspectives

In the past decade, tremendous progress has been made in dissecting the plethora of type III-secreted proteins in plant pathogenic bacteria. Genetic and biochemical studies have led to the identification of a variety of effector proteins that travel the TTS system, the bacterial main road into the host cell. The next major challenge is the functional analysis of effector proteins: what are their targets in the plant and how do they interfere with host cellular processes? Expression of individual effector proteins in plant cells followed by transcriptome analysis and biochemical approaches will advance our understanding of the molecular processes in infected plant cells. Interdisciplinary approaches and comparative analyses of different pathogen–host systems should not only provide

a better understanding of the molecular basis of bacterial pathogenicity but also give us some clues about plant defense and last, but not least, solutions for disease management.

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MINIREVIEW

The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death

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INTRODUCTION

The ability of plant pathogenic bacteria to deliver death-triggering proteins to the interior of plant cells was revealed in a rapid succession of papers in 1996 that transformed our concepts of bacterial plant pathogenicity. The breakthrough came with the convergence of work on Hrp systems and Avr proteins, an understanding of which requires an introduction to the most prevalent bacterial pathogens of plants, the cardinal importance of the Hrp pathway, and the paradoxical phenotype associated with avr genes.

Plant pathogenic bacteria in the genera Erwinia, Pseudomonas, Xanthomonas, and Ralstonia cause diverse, and sometimes devastating, diseases in many different plants, but they all share three characteristics: they colonize the intercellular spaces of plants, they are capable of killing plant cells, and they possess hrp genes. Many of these pathogens are host specific. In host plants, they produce various symptoms after several days of multiplication, whereas in nonhost plants, they trigger the hypersensitive response (HR), a rapid, defense-associated, programmed death of plant cells at the site of invasion (21, 43). With inoculum levels typically encountered in natural environments, the HR produces individual dead plant cells that are scattered within successfully defended healthy tissue (71). However, experimental infiltration of high inoculum levels ($>10^6$ bacterial cells/ml) results in macroscopically observable death of the entire infiltrated tissue, usually within 24 h (42). Pioneer screens for random transposon mutants with impaired plant interactions yielded a prevalent class that was designated Hrp⁻, that is, deficient in both HR elicitation in nonhost plant species and pathogenicity (and parasitic growth) in host species (49, 56). This complete loss of pathogenic behavior results from mutation of any one of several hrp genes, which largely encode components of a type III protein secretion system (73). Because the capacity to elicit the HR is a convenient marker for the capacity to be pathogenic and these two abilities have a common genetic basis, the "simple" problem of HR elicitation is being studied as an entry to the larger problem of pathogenesis.

A key part of the HR puzzle is that HR elicitation and the resulting limitation in host range can occur if the pathogen possesses any one of many possible avr (avirulence) genes that interact with corresponding R (resistance) genes in the host plant. Such "gene-for-gene" interactions result in recognition of the bacterium and the triggering of plant defenses. For example, Pseudomonas syringae pv. glycinea is one of over 40 P. syringae pathovars differing largely in host range among plant

species and is subdivided into races on the basis of their interactions with genetically distinct cultivars of its host, soybean. Those race-cultivar interactions involving matching bacterial avr and plant R genes result in the HR and avirulence, i.e.; failure of the bacterium to produce disease. The R genes encode components of a parasite surveillance system and are crossed into crops from wild relatives by plant breeders for disease control. avr genes are identified and cloned on the basis of the avirulence they confer on virulent races in appropriate test plants (39, 69). In most cases, it is not clear why plant pathogens carry avr genes that betray them to host defenses but new insights into this question are discussed below.

Both hrp and avr genes were originally defined on the basis of the phenotypes they confer on bacteria interacting with plants. Molecular studies have revealed a functional relationship between the products of these two classes of genes and an underlying similarity with a key virulence system of several animal pathogens. Yersinia, Salmonella, and Shigella spp. transfer virulence effector proteins directly into animal cells via the type III pathway (16, 17, 62, 67, 84). Similarly, plant pathogens use the Hrp type III pathway to transfer Avr effector proteins to the interior of plant cells. The genetic dissection of type III secretion systems is just beginning, and little is known of the mechanisms of protein translocation. In this review, we will describe (i) the recently completed inventory of genes directing type III secretion in plant pathogens and new insights into type III secretion mechanisms gained from research with Hrp systems, (ii) two classes of proteins (harpins and pilins) that are secreted by the Hrp type III pathway when plant pathogens are grown in media that mimic plant intercellular fluids, (iii) evidence that Avr proteins are delivered by the Hrp pathway directly to the interior of plant cells, and (iv) a resulting new paradigm for bacterial plant pathogenicity. The focus will be on quite recent work, and readers are referred to other reviews for a classic introduction to the HR phenomenon (43), earlier investigations of the Hrp system (11), avr genes (20, 46), and a wider perspective on bacterial virulence systems and plant responses (2).

Hrp PROTEIN SECRETION SYSTEM

hrp and hrc genes. hrp genes have been extensively characterized in four representative gram-negative plant pathogens: P. syringae pv. syringae (brown spot of bean), Erwinia amylovora (fire blight of apple and pear), Ralstonia (Pseudomonas) solanacearum (bacterial wilt of tomato), and Xanthomonas campestris pv. vesicatoria (bacterial spot of pepper and tomato). Most of the known hrp genes in these strains are contained in chromosomal clusters of about 25 kb (Fig. 1). In at least some cases, the hrp clusters are sufficient to allow HR elicita-
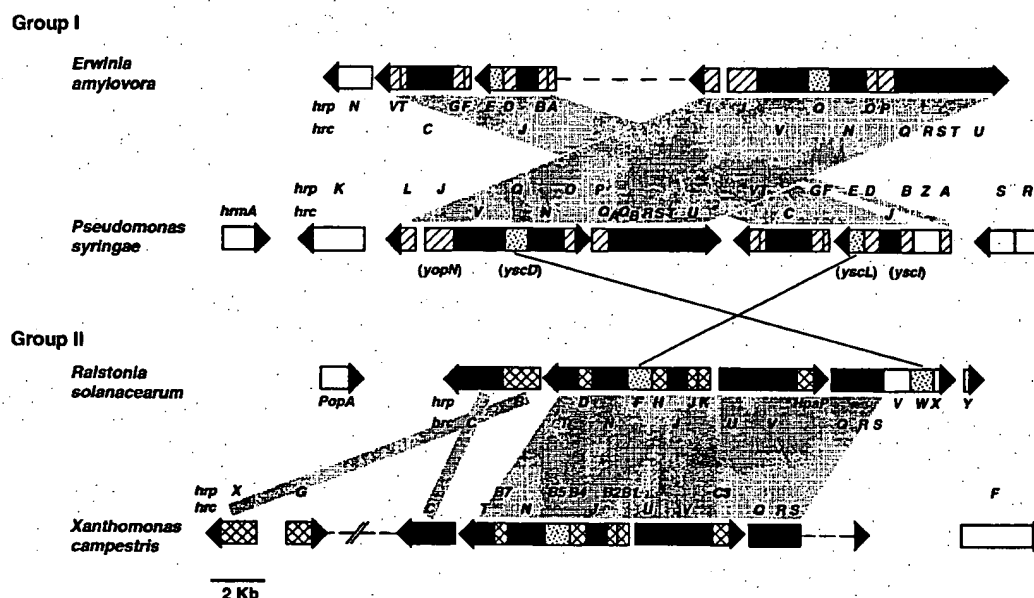
FIG. 1. *hrp* gene clusters of four model plant pathogens. The distribution of each gene among group I and II *hrp* clusters is indicated as follows: *hrc* genes, dark shading; *hrp* genes that are conserved between groups I and II but show weaker similarity to *Yersinia ysc* genes than *hrc* genes, stippling (the two lines between groups indicate homologs); genes common to group I, diagonal lines; genes common to group II, hatching; genes for which no homologs have been reported, white. Dashed lines indicate gaps in the reported sequence of each *hrp* cluster. The shaded bands between members of a group indicate colinear gene arrangements. Note that homologous *hrp* genes have the same designation within group I but not within group II. *Yersinia* genes for which similarity has been noted with *hrp* genes of *R. solanacearum* (74), *E. amylovora* (10, 41), and/or *P. syringae* (36, 60) are in parentheses below the pair of group I *hrp* clusters. The *hrp* cluster of *R. solanacearum* is carried on a megaplasmid (12), but the others appear to be chromosomal. See reference 9 for previous designations of *hrc* genes and for references to all but the recent sequence reports in references 38, 41, and 81.

tion (but not disease) by nonpathogenic bacteria such as *Escherichia coli* and *Pseudomonas fluorescens* (8, 37).

Initial sequencing of the *hrp* clusters from *R. solanacearum*, *X. campestris* pv. vesicatoria, and *P. syringae* pv. syringae revealed homologies with components of the virulence protein (Yop) secretion system of *Yersinia* spp. (22, 29, 34), thereby suggesting the existence of a conserved "type III" protein secretion pathway in gram-negative pathogens of both plants and animals (65, 73). The near completion of these sequences has revealed further homologies and has led to two major changes in the nomenclature of *hrp* genes (9). First, those *hrp* genes that are broadly conserved in pathogenic *Pseudomonas*, *Erwinia*, *Ralstonia*, *Xanthomonas*, *Yersinia*, *Salmonella*, and *Shigella* spp. were redesignated *hrc* (HR and conserved) and given the last-letter designations of their *Yersinia ysc* homologs. The designations for Hrc homologs in various bacteria outside of the plant pathogen group are presented in Table 1. When referred to broadly, the term "*hrp* genes" is intended to encompass the *hrc* subset (9). Second, the *hrp* gene concept was widened to include homologous genes in plant pathogens where mutations do not lead to typical Hrp phenotypes. For example, mutations in *hrp* homologs result in loss of the Wts (watersoaking) phenotype in *Erwinia stewartii* (Stewart's wilt of corn) and reduced infectivity at low inoculum levels in *Erwinia chrysanthemi* (bacterial soft rot) (6, 23). Thus, the *hrp* genes appear to be universal among plant pathogenic *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas* spp. and they control a variety of bacterium-plant interaction phenotypes in addition to the HR.

Group I and II *hrp* clusters. The four *hrp* clusters that have been most characterized can be divided into two groups based on their possession of similar genes, operon structures, and regulatory systems (2). The *hrp* clusters of *P. syringae* and

E. amylovora are in group I, and those of *R. solanacearum* and *X. campestris* are in group II. In addition to the nine *hrc* genes, two *hrp* genes are conserved between the group I and II *hrp* clusters and show some similarities to *ysc* genes (Fig. 1) (10, 36, 41, 74). It is likely that more of the present *hrp* genes will be discerned as belonging to the *hrc* category with additional data on the structure, function, and conservation of their products in both plant and animal pathogens. Nevertheless, some of the *hrp* genes appear to be completely different between the two groups, the arrangements of genes within some operons are characteristic of each group, and the regulatory systems are distinct (Fig. 1). A key difference in regulation is that group I *hrp* operons are activated by HrpL, a member of the ECF (extracytoplasmic function) subfamily of sigma factors (50, 78, 85), whereas most group II *hrp* operons are activated by a

TABLE 1. Hrc proteins of plant pathogenic bacteria and their animal pathogen and flagellar homologs

Plant pathogen protein[a]	Yersinia protein	Salmonella protein	Shigella protein	Flagellar protein(s)
HrcC	YscC	InvG	MxiD	
HrcJ	YscJ	PrgK	MxiJ	FliF
HrcN	YscN	SpaL	Spa47	FliI
HrcQ	YscQ	SpaO	Spa33	FliN, -Y
HrcR	YscR	SpaP	Spa24	FliP
HrcS	YscS	SpaQ	Spa9	FliQ
HrcT	YscT	SpaR	Spa29	FliR
HrcU	YscU	SpaS	Spa40	FlhB
HrcV	LcrD	InvA	MxiA	FlhA

[a] References for the sequences of *hrc* genes and all homologs are compiled in references 9, 25, and 74.

member of the AraC family, which is designated HrpB in R. solanacearum and HrpX in X. campestris (27, 57, 82). However, hrp genes in both groups are generally repressed in complex media and expressed in plants and in media that mimic plant intercellular fluids (11).

Functions of Hrp and Hrc proteins in type III protein secretion. With the hrp clusters of four representative plant pathogens now almost completely sequenced, analysis of the functions of individual components is beginning. Nonpolar mutations have been constructed in most of the hrp and hrc genes in R. solanacearum and in some of the genes in P. syringae pv. syringae and E. amylovora (10, 15, 54, 77). The results suggest that the secretion apparatus requires all of the hrc genes (hrcQ awaits testing). The R. solanacearum mutant analysis also reveals a requirement for hrpF, -W, -K, and -X (54). As discussed above, hrpF and hrpW have group I and possible ysc homologs. Thus, the Hrp type III secretion apparatus is likely composed of a core of 13 proteins, all but 2 of which appear to be broadly conserved. The predicted locations and functions of most of these proteins have been systematically presented for the R. solanacearum Hrp system (74), and they appear to be the same in X. campestris, E. amylovora, and P. syringae.

Sequence comparisons reveal that all of the Hrc proteins, other than HrcC, have a homolog involved in flagellum-specific export or early events in flagellum biogenesis (Table 1). The abilities of the presumably more ancient flagellar system to regulate the order (and possibly amount) of protein released and to secrete proteins in association with an extracellular appendage are properties that may be particularly important in the type III transfer of virulence proteins into host cells (18, 52). Plant pathogens offer several experimental advantages for exploring mechanisms of type III secretion and, indirectly, flagellum-specific secretion. The flagellum-specific and animal pathogen type III secretion systems have been difficult to study because many mutations pleiotropically disrupt production of the secretion apparatus and the secreted proteins. For example, the Yersinia pestis LcrD and Bacillus subtilis FlhA proteins (homologs of HrcV) were initially thought to have primary functions in regulation (14, 59). However, the unambiguous secretion phenotype of an E. amylovora hrcV mutant provided strong evidence that the primary function of members of this protein superfamily is in secretion (77). Plant pathogens offer other experimental advantages for exploring type III secretion mechanisms: defined subclones of ca. 25 kb are conveniently sufficient for Hrp-mediated secretion by E. coli and other model bacteria (31, 77), and hrc gene arrangements and mutant phenotypes suggest that translocation across the inner and outer membranes is partially separable in these bacteria (15).

In both group I and II hrp clusters, the six hrc genes predicted to encode a flagellum-derived system for Sec-independent translocation across the inner membrane (hrcN, -R, -S, -T, -U, and -V) are in operons other than that containing the one hrc gene predicted to direct translocation across the outer membrane (hrcC) (Fig. 1 and 2). HrcC is a member of the PulD/pIV superfamily of outer membrane proteins, which are involved in type II protein secretion (PulD) and filamentous phage secretion (pIV) (26). These proteins form homomultimers in the outer membrane which permit phage or protein exit and induce the psp (phage shock protein) operon (63). The HrcC protein of X. campestris pv. vesicatoria was the first member of the type III branch of this superfamily shown to induce the psp operon, thereby suggesting that the type III pathway also employs an outer membrane, channel-forming multimer (80). A P. syringae pv. syringae hrcC mutant accumulates some of the normally secreted HrpZ harpin (discussed below) in the periplasm, whereas a hrcU mutant accumulates
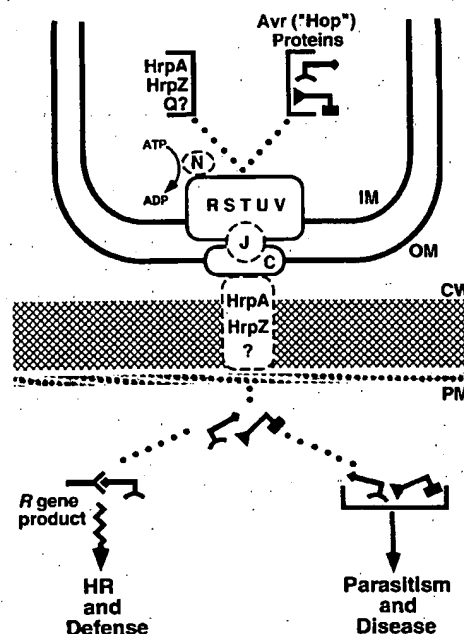

FIG. 2. Model for the delivery of parasite-promoting Avr proteins (i.e., Hop proteins according to a proposal discussed in the text) into plant cells by the Hrp type III secretion system (P. syringae example). To reach their targets, Avr proteins must cross the bacterial inner membrane (IM), outer membrane (OM), plant cell wall (CW), and plasma membrane (PM). Only Hrc components (indicated by their last letters in predicted subcellular locations) and proteins known to be secreted are shown. The location of hydrophilic HrcQ (HrcQ_A and HrcQ_B in P. syringae) is unknown, but the homologous SpaO is secreted by Salmonella spp. (25, 48). Four additional Hrp proteins, not shown, appear to be required for secretion (see text). Dashed-line boxes indicate uncertainties about precise location. For example, it is not known whether HrpA or HrpZ penetrates the plant cell wall and whether these and/or other Hrp proteins trigger Avr transfer into plant cells by endocytosis. Secretion of HrpA and HrpZ is not dependent on plant cell contact, whereas secretion of Avr proteins apparently is. Once inside plant cells, multiple Avr proteins apparently promote parasitism collectively by unknown mechanisms (short arms denote weak phenotypes of virulence domains interacting with undefined host targets), unless any one of the proteins interacts with a host R gene product, thereby triggering the HR defense. Mutation of a host target, to diminish benefit to the parasite, and detection by the R gene surveillance system are likely evolutionary responses of plants to the bacterial deployment of a new virulence protein; coevolution would be expected to generate many avr and R genes in complex populations of plants and bacterial parasites.

the protein exclusively in the cytoplasm (15). Thus, the sequence-based prediction that separate inner and outer membrane translocator systems have been recruited to form the Hrp pathway is supported by a novel secretion phenotype revealing partial separation of these functions (15).

HARPINS, PILINS, AND OTHER PROTEINS SECRETED IN CULTURE BY THE Hrp SYSTEM

Harpins. Broadly defined, harpins are glycine-rich proteins that lack cysteine, are secreted in culture when the Hrp system is expressed, and possess heat-stable HR elicitor activity when they infiltrate the leaves of tobacco and several other plants. As is characteristic of proteins secreted by the type III pathway, harpins lack an N-terminal signal peptide. The first harpin was discovered in the culture fluids of E. coli cells carrying a highly expressed hrp cluster from E. amylovora (79). Because mutations in the harpin-encoding hrpN gene in E. amylovora strongly diminish HR elicitation in tobacco and pathogenicity in susceptible, immature pear fruits, harpin was initially thought to be the primary virulence protein traveling the Hrp pathway

(79). Subsequent analysis of harpins from other bacteria has revealed that harpins differ substantially in their primary structure and their contribution to Hrp phenotypes, and their actual function is unknown (4, 7, 19, 31).

The harpin genes of *E. amylovora* (*hrpN*) (79), *E. chrysanthemi* (*hrpN_Ech*) (7), and *R. solanacearum* (*popA*) (4) are located adjacent to or near their respective *hrp* clusters, whereas the *P. syringae hrpZ* gene resides within a *hrp* operon (31). *E. chrysanthemi hrpN* mutants are reduced in infectivity at low inoculum levels and are unable to elicit the HR (7), but harpin gene mutations in *E. amylovora* CFBP1430 (a highly virulent strain) (5), *R. solanacearum* (4), and *P. syringae* (1) produce weak phenotypes or no phenotype. Thus, individual harpins do not appear to be necessary for elicitation of the HR by most bacteria. The potential role of harpins in determining host specificity is uncertain. PopA may be a host specificity factor because the isolated protein elicits the HR selectively in those plants in which *R. solanacearum* also elicits the HR, whereas the isolated harpins from *E. amylovora* and three *P. syringae* pathovars trigger the HR in various plants in a manner that shows no relationship to bacterial host range (30, 31, 60, 79). Harpin activity may involve interactions with plant cell walls. The HrpZ harpin binds to the walls of intact plant cells but not to protoplasts, and it also fails to trigger HR-associated responses in protoplasts (33). The elicitor activity of harpins is unlikely to be enzymatically based because various fragments retain activity (1, 4, 45).

The function of the *P. syringae* HrpZ harpin is particularly puzzling. Several observations suggest a simple, direct role for HrpZ in HR elicitation. HrpZ is the predominant protein secreted by the *P. syringae* Hrp system in culture (31, 88), the *hrpZ* gene is conserved in divergent *P. syringae* pathovars (60), and the isolated protein elicits an apparent programmed cell death in plants that is indistinguishable from the HR elicited by living bacteria (31). Furthermore, *hrpZ* deletion mutations in the cosmid pHIR11 functional cluster of *P. syringae* pv. syringae *hrp* genes strongly reduce the HR elicitation activity of *E. coli* cells carrying only pHIR11. The same mutation only slightly reduces the HR in *P. syringae* pv. syringae, but this can be explained by postulating the existence of a second harpin encoded elsewhere in the bacterial genome (1).

However, other observations show that the relationship of HrpZ to HR elicitation is more complex. Mutation of *hrmA* (32, 35), which is in a variable region flanking the conserved *hrp* cluster in pHIR11, abolishes HR activity in tobacco without diminishing HrpZ synthesis or secretion (1). Thus, isolated HrpZ is sufficient to elicit an HR in tobacco leaves but HrpZ produced by bacteria in plants is not. Instead, HrmA, with no apparent function in the Hrp secretion apparatus, is necessary for bacterial elicitation of the HR, and thus, HrmA appears to be the actual elicitor of the HR produced by bacteria carrying pHIR11. HrmA has several characteristics of an Avr protein (3). Avr proteins and the role of the Hrp system (and possibly harpins) in their delivery into plant cells will be discussed below.

HrpA pilin and other secreted proteins. *P. syringae* pv. tomato DC3000 secretes at least four proteins in addition to HrpZ into the medium in a Hrp-dependent manner (88). One of these is the 10-kDa product of *hrpA*, which forms a 6- to 8-nm-diameter "Hrp pilus" (61). A nonpolar *hrpA* mutant no longer elicits the HR in appropriate test plants, even when carrying an *avr* gene known to interact with an *R* gene in the plant. It thus appears that the Hrp pilus is essential for the delivery of Avr signals (discussed further below). Although it is not known whether the Hrp pilus functions primarily in bacterial attachment or as a conduit for the delivery of bacterial

proteins across the plant cell wall, it is interesting that *Agrobacterium tumefaciens* requires a pilus similar in size (3.8-nm diameter) to transfer T-DNA and the VirE2 protein into plant cells (24).

Hrp DELIVERY OF AVR PROTEINS INTO PLANT CELLS

avr genes and their products. In fundamental contrast to the *hrp* genes, *avr* genes are scattered in their distribution among strains of plant pathogenic bacteria (20, 46). More than 30 bacterial *avr* genes have been cloned from *P. syringae* and *X. campestris*, but until recently, characterization of the menagerie of encoded proteins has largely defined what these proteins do not do. Isolated Avr proteins do not elicit any responses when they infiltrate plant leaves. They do not appear to be secreted in culture and are hydrophilic proteins lacking N-terminal signal peptides or other recognizable secretion signals (properties consistent with potential secretion by the type III pathway). They do not have demonstrable enzymatic activity (with the exception of AvrD, which directs the synthesis of syringolide elicitors of an *R* gene-dependent HR [55]), and the majority of them do not contribute in an obvious way to parasitic fitness or virulence in the infection of cultivars lacking a matching *R* gene that would trigger the HR. However, there are several significant exceptions to the last point (20, 46) and there is growing evidence that Avr proteins have a primary function in virulence, even though the HR-triggering effects of *Avr-R* interactions are epistatic over these virulence functions. How Avr proteins might promote parasitism is mysterious, but support for such a primary role comes from observations that their action is dependent on the Hrp system and their site of action is within host cells. The next two sections address these points and provide evidence that the main function of the Hrp system is in the delivery of Avr-like proteins into plant cells.

Hrp dependency of Avr phenotypes. *avr* genes have no phenotype when expressed in *hrp* mutant pathogens or in nonpathogenic bacteria like *E. coli*, which lack the Hrp system (highly expressed *avrD* is the sole exception to the latter point [40]). For many *avr* genes, especially those in *P. syringae*, one simple explanation is that their expression is dependent on Hrp regulatory factors (46). However, expression of *avr* genes from vector promoters does not obviate the requirement for a functional Hrp system. The recent finding that the functional cluster of *P. syringae* pv. syringae *hrp* genes carried on cosmid pHIR11 is sufficient to deliver heterologous *avr* gene signals indicates the fundamental interdependency of Hrp and Avr functions in bacterial elicitation of the HR (28, 58). A key property of pHIR11 enabling this discovery is that the cosmid confers on nonpathogenic *E. coli* and *P. fluorescens* the ability to elicit the HR in tobacco and several other plants, but it is ineffective in doing so in soybean and *Arabidopsis*. The simplest explanation is that *hrmA*, which is carried on pHIR11 and has several properties of *avr* genes (3), interacts with an unknown *R* gene in tobacco but with no *R* genes in soybean and *Arabidopsis*. This suggested that expression of appropriate *avr* genes in *trans* would enable nonpathogens carrying pHIR11 to elicit an *R* gene-dependent HR in soybean, *Arabidopsis*, and other plants. Indeed, this was observed with *avrB* (from *P. syringae* pv. glycinea) and five other *P. syringae avr* genes (28, 58).

The ability of pHIR11 to deliver *avr* gene signals requires HrcC (absolutely) and HrpZ (variably) (28, 58). The inability of HrpZ to support AvrB signal delivery when supplied exogenously indicates that the harpin has a role only when produced along with AvrB and therefore may be an extracellular accessory in the delivery of Avr proteins, as YopD is in the

delivery of YopE (28, 62). Most importantly, these experiments reveal that a functional Hrp secretion system is required for the delivery of several avr gene signals. Furthermore, the use of promoters different in strength and of epitope-tagged AvrB revealed that the requirement for a functional Hrp secretion system cannot be obviated by high levels of AvrB in the bacterial cytoplasm or by infiltration of leaves with purified AvrB at a level 1,000-fold higher than that required by living Hrp⁺ bacteria to elicit the HR (28). Thus, AvrB does not appear to act in the bacterial cytoplasm or in leaf intercellular spaces. These observations strongly support the hypothesis, depicted in Fig. 2, that the type III protein secretion system in plant pathogens, as in animal pathogens, is capable of delivering bacterial proteins into host cells.

Demonstrations of Avr action in host cells. Bacterial transfer of Avr proteins into plant cells has not been observed directly. However, there is evidence that several of these proteins are biologically active when produced within plant cells, that the HR-triggering activity of one of them is dependent on physical interaction with its cognate plant R gene product, and that the activity of another is dependent on localization to the plant cell nucleus. AvrB action in plant cells was demonstrated with Arabidopsis plants carrying the cognate RPM1 R gene (28). An Arabidopsis rpm1 mutant was transformed with avrB and crossed with a wild-type line, thus producing seedling progeny carrying both avrB and RPM1 which died soon after germinating. One symptomless rpm1 mutant transgenic plant was obtained; this individual expressed relatively low levels of an avrB construct carrying the PR-1a plant protein signal peptide, with the likely consequence that the plant cytoplasm would be exposed only transiently or to low levels of AvrB. The properties of this survivor suggest that plants are sensitive to AvrB even in the absence of a functional matching R gene and that vanishingly low levels of the protein are sufficient to elicit the HR in the presence of a complete R gene. A biolistic, transient expression assay revealed that avrB lacking a signal peptide (and therefore localized to the plant cytoplasm) was lethal to Arabidopsis leaf cells carrying RPM1 but not to those lacking the R gene (28). This approach was extended with avrRpt2 (from P. syringae pv. tomato) (47). Similarly, an A. tumefaciens transient expression system was used to deliver avrPto (from P. syringae pv. tomato) and avrBs3 (from X. campestris pv. vesicatoria) into plants, resulting in an R gene-dependent HR in all cases (66, 70, 72). Thus, whereas no bacterial Avr protein has been observed to have an effect when delivered exclusively to the surface of plant cells, all four of those tested elicit an R gene-dependent response when expressed inside them.

The simplest model for the molecular basis of gene-for-gene HR elicitation predicts physical interaction between the protein products of cognate avr and R genes. This has been observed with the bacterial AvrPto and plant Pto proteins; mutations in the molecular partners that diminish physical interaction in the yeast two-hybrid system also diminish biological function (66, 70). Because AvrPto action requires a functional Hrp system in either P. syringae pv. tomato (64) or nonpathogens carrying the pHIR11 hrp cluster (28, 58) and it involves physical interaction with a cytoplasmic target in the host, the Hrp-mediated transfer of AvrPto into plant cells seems certain.

While many bacterial Avr proteins appear to be targeted to the host plant cytoplasm, members of the AvrBs3 family in Xanthomonas spp. are targeted to the host nucleus. These proteins carry functional nuclear localization signals (NLS) in the C-terminal region (72, 86). When fusions of this C-terminal region and a uidA reporter are transiently expressed in onion epidermal cells by biolistic bombardment, β-glucuronidase ac-

tivity is localized to the nucleus (72, 86). Deletion of all three of the NLS sequences abolishes nuclear localization in the biolistics assay and HR elicitation by X. campestris pv. vesicatoria cells in pepper plants carrying the Bs3 R gene, and both of these abilities can be restored by substitution of the simian virus 40 large-T antigen NLS (72). These results suggest that the Bs3 product must also be localized to the nucleus, but because this R gene has not been cloned, this awaits confirmation.

Gaps in our knowledge of the Hrp pathway and the inventory of its protein traffic. Although the rings of evidence that the Hrp system transfers Avr proteins into plant cells are collectively strong, there are formal gaps in each. (i) In the system explored in the most detail, AvrPto-Pto, physical interaction between the bacterial and plant proteins has not been demonstrated in vivo, and a second host protein, Prf, is required for AvrPto-Pto-mediated HR elicitation. Furthermore, all of the other cloned plant R genes that interact with known bacterial avr genes resemble Prf (a nucleotide-binding site leucine-rich repeat protein) rather than Pto (a kinase) (68). (ii) R proteins appear to be present at vanishingly low levels, and none has been directly observed in the cytoplasm, although RPS2 localizes to the cytoplasm-equivalent fraction in a rabbit reticulocyte dog pancreatic microsome in vitro translation-translocation system (47). (iii) Similarly, Avr proteins appear to be effective at vanishingly low levels (28) and immunogold labeling and electron microscopy of infected plant tissues has revealed their presence only in bacterial cells (13, 87). (iv) Finally, no Avr protein has been directly shown to be translocated out of the bacterial cytoplasm in culture by the Hrp system. It is worth noting that the A. tumefaciens VirE2 protein has never been observed to be transferred into plant cells, although the indirect evidence for its action within plant cells seems irrefutable (89).

Many (if not most) of the genes encoding proteins that are transferred into plant cells by these bacterial pathogens probably await discovery. Systematic completion of the inventory is thwarted by two problems. First, the contribution of the genes to virulent interactions may be too subtle for detection in mutant screens, and cognate R genes that would reveal Avr phenotypes when the bacterial genes are heterologously expressed may be unknown or nonexistent. Second, no plant signals or regulatory mutants have been found that permit bacteria to secrete these proteins in culture, although harpins, pilins, and possibly other proteins that serve the type III secretion system are secreted in culture. A critical feature of the type III protein secretion system in Yersinia spp. is its capacity to withhold full secretion of virulence proteins until contact with the host cell (18). The fact that nonpathogens carrying the pHIR11 functional hrp cluster secrete HrpZ but not AvrB in culture (28) indicates that the genetic information for this expected regulatory step is carried within the hrp cluster and is therefore subject to discovery through systematic analysis of the hrp genes. Obtaining Avr protein secretion in culture is important because (i) it is likely to be associated with structures that normally are used to penetrate the plant cell wall (and possibly trigger host cell endocytosis) and therefore will yield clues to the transfer process and (ii) it will allow proteins targeted to the host to be systematically characterized through identification of novel proteins in the medium. The exploration of DNA sequences flanking hrp clusters also should be useful in this search because of the growing evidence that these regions are enriched in genes whose products probably travel the Hrp pathway (51, 53, 54).

A new designation for effector proteins that are delivered by the Hrp system to plant cells would be useful: Avr appears

to be inappropriate because some of the encoding genes may have no Avr phenotype and the primary function of Avr proteins is almost certainly in virulence, not avirulence. One proposal is to designate new members of this class Hop (Hrp-dependent outer protein) and to add a four-letter suffix identifying the bacterial species, pathovar, and gene, based on the current system for uniform nomenclature of avirulence genes (3, 75). For example, the gene encoding a newly found *P. syringae* pv. syringae protein in this class would be designated *hopPsyA*. Hop is analogous to the Yop (Yersinia outer protein) designation for proteins secreted by the prototypical *Yersinia* type III secretion system but is broadened here for consistency with the use of Hrp and Avr for plant pathogens in all genera.

A NEW PARADIGM AND FUTURE EXPLORATIONS

Pathogenesis based on the Hrp delivery of Avr-like (Hop) proteins into host cells (depicted in Fig. 2) provides a simple and unifying explanation for many characteristics of plant pathogenic *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia* spp. (2). These include the one-to-one relationship between bacterial cells and HR-responding plant cells (expected with contact-dependent secretion), the gene-for-gene interactions of pathogen races and host cultivars (expected if *avr* and *R* gene products can directly interact within host cells), and the enormous diversity in host range and other pathogenic attributes among closely related strains (expected with a pool of horizontally transferable and interchangeable genes whose products can either promote or betray parasites in coevolving hosts). The latter point is particularly relevant to *P. syringae* and *X. campestris*, which are divided into more than 40 and 140 pathovars, respectively. And it is consistent with the location of many *avr* genes on plasmids and the ability of *avr* genes to function with heterologous Hrp systems (20). In this regard, one potential difference between the type III systems of animal and plant pathogens is noteworthy. In animal pathogen type III systems, the secretion of many effector proteins requires customized chaperones, which are often encoded by genes linked to effector genes (76). The ability of many isolated *avr* genes to function heterologously in other pathogens or in nonpathogens carrying the pHIR11 functional *hrp* cluster suggests that Avr protein delivery does not require specific chaperones or that a promiscuous chaperone gene exists within the *hrp* cluster.

This new model of plant pathogenicity invites several fundamental questions in plant pathology and pathogenic microbiology in addition to those discussed above regarding the Hrp system and the identification of its traffic. How do Hrp-delivered proteins alter host metabolism to promote bacterial growth in plant intercellular spaces? How is host specificity determined at the pathovar-host species level? That is, are *avr-R* gene interactions important here also, as suggested by the discovery of novel *avr* genes through expression in heterologous pathovars (44, 83), or do Avr-like proteins have important positive effects in bacterial adaptation to host species? Given the use of homologous secretion systems, how similar are the functions of the virulence proteins that plant and animal pathogens transfer into their hosts? Sequence similarities involving secreted *Yersinia* proteins have been noted only between YopN and YopJ and the *E. amylovora* HrpJ and *X. campestris* pv. vesicatoria AvrRxv proteins, respectively (10, 46). Since YopN appears to be an extracellular component of the secretion system and the effector activity of YopJ is unknown, this key question remains unanswered. Further comparisons should give us a broader perspective on the evolution of bacterial pathogenicity and may lead to unanticipated controls for diseases of both plants and animals.

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Review

Are elicitins cryptograms in plant-Oomycete communications?

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Abstract. Stimulation of plant natural defenses is an important challenge in phytoprotection prospects. In that context, elicitins, which are small proteins secreted by *Phytophthora* and *Pythium* species, have been shown to induce a hypersensitive-like reaction in tobacco plants. Moreover, these plants become resistant to their pathogens, and thus this interaction constitutes an excellent model to investigate the signaling pathways leading to plant resistance. However, most plants are not reactive to elicitins, although they possess the functional signaling pathways involved in tobacco responses to elicitin. The understanding of factors involved in this reactivity is needed to develop agronomic applications. In this review, it is proposed that elicitins could interact with regulating cell wall proteins before they reach the plasma membrane. Consequently, the plant reactivity or nonreactivity status could result from the equilibrium reached during this interaction. The possibility of overexpressing the elicitins directly from genomic DNA in *Pichia pastoris* allows site-directed mutagenesis experiments and structure/function studies. The recent discovery of the sterol carrier activity of elicitins brings a new insight on their molecular activity. This constitutes a crucial property, since the formation of a sterol-elicitin complex is required to trigger the biological responses of tobacco cells and plants. Only the elicitins loaded with a sterol are able to bind to their plasmalemma receptor, which is assumed to be an allosteric calcium channel. Moreover, *Phytophthora* and *Pythium* do not synthesize the sterols required for their growth and their fructification, and elicitins may act as shuttles trapping the sterols from the host plants. Sequence analysis of elicitin genes from several *Phytophthora* species sheds unexpected light on the phylogenetic relationships among the genus, and suggests that the expression of elicitins is under tight regulatory control. Finally, general involvement of these lipid transfer proteins in the biology of Pythiaceae, and in plant defense responses, is discussed. A possible scheme for the coevolution between *Phytophthora* and tobacco plants is approached.

Key words. Elicitin; cell death; hypersensitivity; LTP; *Phytophthora*; *Pythium*; resistance; SAR; SCP; signaling; tobacco.

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Introduction

Crop protection constitutes a major challenge in both improving produce quality and preserving the environment. Interactions between microorganisms and plants have had major effects on the development of civilizations. Plant disease outbreaks have resulted in catastrophic crop failures and caused major social changes. However, disease is not the only outcome of plant-microbe interactions, and biological controls of plant disease are emerging, using molecules from pathogens able to induce defense mechanisms in plants (for a recent overview of plant-microbe interactions see [1]). In some situations, microorganism molecules could be recognized by plant cells, triggering many metabolic changes and leading to plant resistance. Such compounds have been named elicitors of plant defense and have been extensively studied [2–8].

The hypersensitive response (HR) is the most common feature associated with active plant resistance. It follows a primary pathogen attack. Activation of the HR leads to the death of cells at the site of infection, resulting in the restriction of the pathogen to small areas surrounding the initially infected cells (necrotic lesions). In plant resistance associated with HR, the knowledge of events which trigger cell death is fundamental. In some situations, the development of HR is followed by the induction of a systemic acquired resistance (SAR). The whole plant becomes resistant to further pathogen infection, wherever this infection occurs. SAR activation results in the development of broad-spectrum resistance. A strong body of evidence suggests that salicylic acid plays a key role in both SAR signaling and disease resistance [9–12].

In tobacco fields, it was shown that necroses on plants were associated with the presence of Phytophthora, which were nonpathogenic [13]. From culture filtrates of P. cryptogea and of P. capsici, proteinaceous elicitors named elicitins (cryptogein and capsicein, respectively) were subsequently isolated [14]. These proteins stimulate natural defenses of tobacco against many pathogens, accompanied by restricted leaf necrosis [15–17]. About 40 Phytophthora species were screened for the production of elicitins, and different modes of treatment were assayed for plant responses [18–20]. Using cryptogein antibodies, it was shown that this elicitin could migrate through the plant and could be responsible for the systemic acquired resistance induced in tobacco [21–23]. A possible extension towards other plants could be offered with another genus of the oomycete class, Pythium spp., which can induce protection of tomato against Fusarium oxysporum f. sp. radicis-lycopersici [24, 25] and can also secrete elicitin-like proteins.

This paper emphasizes recent results concerning elicitins and reflects on about their agronomic interest and biological functions. Recent contributions bring responses to some of the problems still unsolved after publication of the last reviews on that exciting model [26–28]. Until now, elicitins were viewed only as elicitors of plant defenses. The biological functions of these holoproteins remained unknown, although the genes coding elicitins, despite strong selection pressure, were highly conserved and seemed tightly regulated. The discovery of their sterol carrier properties has opened new perspectives dealing with the relationship between this function and the elicitor activity of these small cysteine-rich proteins. Nevertheless, this elicitor activity was restrained to few plant species, and thus did not appear to be in accordance with a universal lipid transfer function. These considerations required a reassessment of the precise role of elicitins for both Phytophthora and plants.

Elicitins, a family of (not so?) well-characterized proteins

Elicitins can be merged within a single, highly conserved, family. Purified proteins as well as numerous sequences derived from complementary DNA (cDNA) clones are similar enough to fit this classification. On the other hand, elicitins do not resemble any other proteins, and previous attempts to compare them with other small, fungal proteins, such as hydrophobins, were poorly convincing and have been abandoned [26]. So what does allow the assignment of a given protein to the elicitin family from a structural point of view?

First of all, elicitins are restricted to the oomycete genus Phytophthora and a few Pythium species, which, on the basis of additional taxonomic features may be missing links between the two genera [29]. Oomycetes are no longer considered to be 'fungi', but have been phylogenetically grouped with the heterokont algae [30]. Moreover, the other families of Oomycetes appear to lack elicitin genes or equivalent sequences [30]. Thus, elicitins are located in a particular, unexplored, taxonomical niche. This may explain the paucity of comparative models available in plant (or animal) pathology.

Additional features that make a protein an elicitin are the sum of some characteristics: the size (98 amino acids in most cases); a biased amino acid composition, revealed by the lack of tryptophan, histidine and arginine residues and the significant abundance of a few amino acids, such as serine and threonine residues, that represent about 30% of the protein, and to a lesser extent alanine (more than 10%) and leucine (10%); the occurrence of six cysteine residues located on conserved positions and involved in three structurally determinant disulfide bridges, and an overall primary structure which represents a unique amino acid sequence that

enables definition of an 'elicitin signature' listed in the various pattern databases such as PRODOM [31] or PRINTS [32] and that spans the entire sequence (fig. 1). In addition, the lack of tryptophan implies that elicitins display typical, tyrosine-like ultraviolet (UV) spectra, giving a helpful signature for their characterization among other proteins. Finally, a protein would be also defined as an elicitin on the basis of its particular three-dimensional structure [33] and biophysical properties (see below).

To date, more than 30 *Phytophthora* species have actually been found to secrete elicitins. Based on their respective pI, all these proteins could be classified as either acidic (α, pI < 5) or basic (β, pI > 7.5) elicitins. These two forms could be encountered within the same *Phytophthora* species, but an iterative rule could be

pointed out: α-elicitins were always produced, whereas β-elicitins were found to be secreted by a restricted range of species. The biological and taxonomical relevance of this situation will be discussed later.

A rapid comparison of α- and β-elicitins led to identification of proper characteristics for each form. First, the evident difference proceeds from the global net charge due to differing composition in charged amino acids; the positive charge is provided by 6 Lys in β-elicitins, and only 2–4 in α-elicitins. In contrast, the number of negatively charged Asp and Glu residues was almost constant within the proteins, ranging from 3 to 5, and was not correlated with resulting net charge. Second, structural differences were reported among the amino acid sequences that were not directly related to protein net charge. For example, in β-elicitins, residues 13 and

```
cinnamomin-ha1    TACTSTQQTAAYVALVSILSESYFSTCASDSGYSMLTATALPTTAQYKLMCASTACQEMI
hae1-cryptogein   TACTTTQQTAAYVALVSILSESFFSTCASDSGYSMLTATALPTTAQYELMCASTACQEMI
hae2-cryptogein   TACTTTQQTSAYVTLVTLLSKSYFTTCASDSGYSMLTATALPTTAQYELMCASTACQEMI
cinnamomin-ha2    TACTSTQQTAAYVALVSILSESYFSTCASDSGYSMLTATALPTTAQYKLMCASTACQEMI
ß-cryptogein      TACTATQQTAAYKTLVSILSDASFNQCSTDSGYSMLTAKALPTTAQYKLMCASTACNTMI
ß-cinnamomin      TACTATQQTAAYKTLVSILSESSFSQCSKDSGYSMLTATALPTNAQYKLMCASTACNTMI
parasiticeinA1    TTCTTTQQTAAYVALVSILSDTSFNQCSTDSGYSMLTATSLPTTEQYKLMCASTACKTMI
infestin1         TTCTTSQQTVAYVALVSILSDTSFNQCSTDSGYSMLTATSLPTTEQYKLMCASTACKTMI
α-cryptogein      TTCTTTQQTAAYVALVSILSDSSFNQCATDSGYSMLTATSLPTTDQYKLMCASTACNSMI
α-cinnamomin      TTCTSTQQTAAYVALVSILSDSSFSQCATDSGYSMLTATSLPTTAQYKLMCASTACNTMI
sojein2           TTCTSSQQTAAYVALVSILSDSSFNQCATGSGYSMLTATALPTTAQYKLMCASTACNTMI
infestin2A        ETCSPTDQTTAYSTLASVLTLSSFQGCADDSGFSLLYSTALPDDDQYVKMCASDNCKSLI
infestin2B        ETCSPTDQTTAYSTLASVLTLSSFQGCADDSGFSLLYSTALPDDAQYVKMCASDNCKSLI
                  :*:..:** ** :*.::*: : *  *: .**:*:* :..** ** **** *: :*

cinnamomin-ha1    TEIIALDPPDCDLTVPTSGLVINVYEYSNDFVSTCASLSSSASS----------------
hae1-cryptogein   EEIIALNPPDCDLTVPTSGLVINVYEYANDFASTCASLSSSPA-----------------
hae2-cryptogein   AEIITLSPPDCDLTVPTSGLVIDVYTYANGFASTCASLSSSSA-----------------
cinnamomin-ha2    TEIVSLSPPDCDLTVPTSGLVLDVYTYANGFTLTCASLSSSSA-----------------
ß-cryptogein      KKIVTLNPPNCDLTVPTSGLVLNVYSYANGFSNKCSSL----------------------
ß-cinnamomin      KKIVALNPPDCDLTVPTSGLVLDVYTYANGFSSKCASL----------------------
parasiticeinA1    NKIVSLNPPDCELTVPTSGLVLNVFTYANGFSSTCASL----------------------
infestin1         NKIVSLNAPDCELTVPTSGLVLNVYSYANGFSSTCASL----------------------
α-cryptogein      AKIISLNAPDCELTVPTSGLVLNVYSYANGFSATCASL----------------------
α-cinnamomin      KKIVTLNPPDCELTVPTSGLVLNVYSYANGFSATCASL----------------------
sojein2           KKIVALNPPDCDLTVPTSGLVLNVYSYANGFSSTCASL----------------------
infestin2A        ESVASLNPPNCDLTVPTSGLVLNVVDLTSGFSEKCSSSSSTSNTASSAATSTTTEAPAAT
infestin2B        ESVAGLNPPNCDLTVPTSGLVLNVVDLTSGFSKKCSSSSSTSNTASSAATSRTTEAPAAT
                  .: *..*:*:*********::* :..* .*:*

cinnamomin-ha1    -----------------------------------------------
hae1-cryptogein   -----------------------------------------------
hae2-cryptogein   -----------------------------------------------
cinnamomin-ha2    -----------------------------------------------
ß-cryptogein      -----------------------------------------------
ß-cinnamomin      -----------------------------------------------
parasiticeinA1    -----------------------------------------------
infestin1         -----------------------------------------------
α-cryptogein      -----------------------------------------------
α-cinnamomin      -----------------------------------------------
sojein2           -----------------------------------------------
infestin2A        TAAPTTDTTTASTATDAPAATPVATPATTTTNSTESVTQTS----AAAC
infestin2B        TAAPTTDSTATDAPAATPAVTPVATPAATSTNSTESVTQTSVTQTTAAC
```
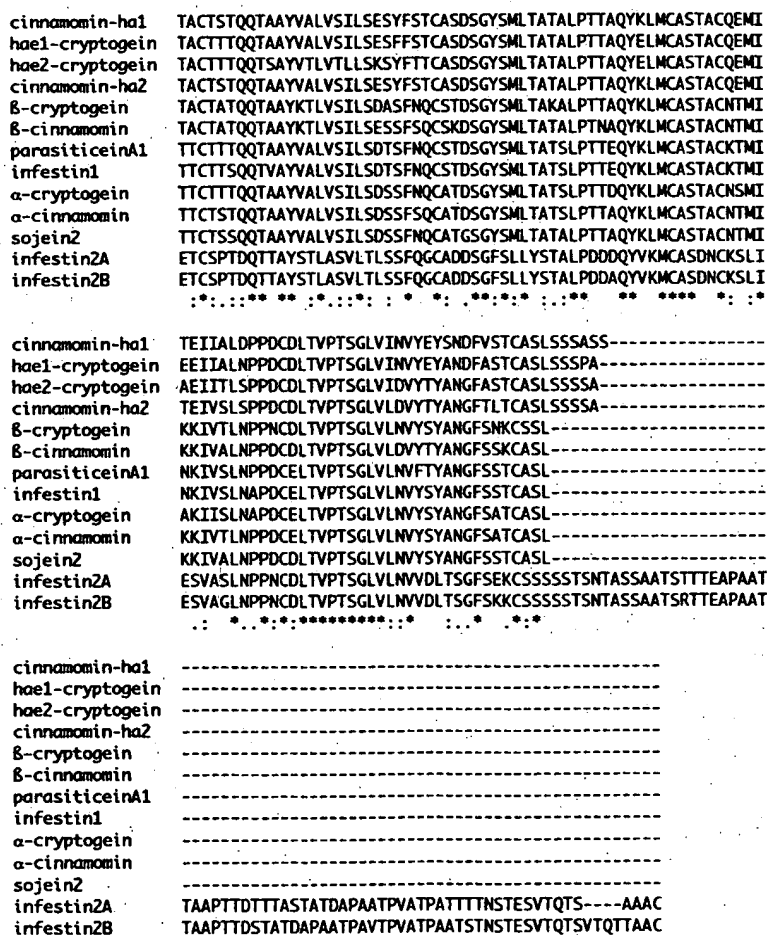
Figure 1. Clustal W multiple sequence alignment of elicitins. (*) fully conserved residue and (:) conserved strong group; (.) conserved weaker group.
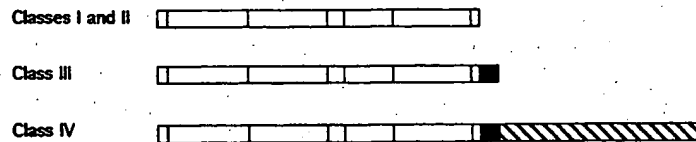
Figure 2. Schematic representation of the various elicitin classes. The 'elicitin' domain, as defined in the PRODOM [31] and PRINTS [32] databases, is represented by open rectangle. Vertical bars indicate the location of conserved cysteine residues. The carboxy-terminal extension shared by the genes encoding highly acidic elicitins (HAE) and elicitin-like proteins from *P. infestans* is represented by a black square, whereas the carboxy-terminal regions specific to class III are indicated by a dashed rectangle.

14 are polar amino acids (Lys, Thr), which constitute an interruption in the hydrophobic segment A(V)AYVA(T)LV, compared with α-elicitin. It is noticeable that a chemical characteristic (pI) revealed structural coherences with evident links to gene structure, so that β-elicitins appeared as original proteins not only because of their presence in few species of *Phytophthora* but also according to their sequence.

3D structure of cryptogein

The three-dimensional (3D) structure of the β-elicitin cryptogein was determined by X-ray diffraction of the protein crystal [33] and by ^{1}H and ^{15}N nuclear magnetic resonance (NMR) of protein solution [34, 35]. These structures are closely related, and only slight differences occur. The crystal structure is probably more compact, whilst the solution structure highlighted the flexibility of the protein. Cryptogein is a globular protein containing five helices (α_1–α_5, from N- to C-term, respectively), a small antiparallel β-sheet and an Ω-loop, all these structures being linked together by disulfide and hydrogen bonds. The disulfide bridges were characterized as follows: Cys3–Cys71, Cys27–Cys56 and Cys51–Cys95 [33, 36, 37]. Roughly, the protein shows two opposite sides: on one side α-helices making a smooth face with polar amino acids exposed to the solvent, and on the other side a protruding beaklike motif built from the proximity of the Ω-loop and the β-sheet (see fig. 10). A large hydrophobic cavity occurs between these two faces, mainly involving strictly or highly conserved hydrophobic residues (Met, Leu, Ile, Phe, Val, Tyr). From the solution structure, it appears that the Ω-loop is flexible. The tightening of Ω-loop and β-sheet is secured by hydrogen bondings and van der Waals interactions. The study of conformational changes, due to pH and elicitin concentration, demonstrates that cryptogein is able to dimerize [35].

Distinct classes of elicitins

The further identification of numerous cDNA clones from various *Phytophthora* species [F. Panabières et al.,

unpublished results], as well as genomic clones containing elicitin-encoding sequences in *P. cryptogea* [38] *P. cinnamomi* [39] and border species of *Pythium* [29], revealed additional, unusual features, especially on elicitin genes, which appear not to be expressed [38]. Thus elicitin-encoding sequences can be separated (to date) into three broad classes as follows (figs 1 and 2):

● Class I encompasses all the proteins (or open reading frames, ORFs) corresponding to the elicitin type, exhibiting most of the features described above. In addition, the totally conserved residues are the 6 Cys, 3 Met, 2 Phe and 3 Gly. Residues Leu, Ile, Pro and Thr are always located on highly (or strictly) conserved positions.

● Among this class, one group (I') corresponds to elicitins which slightly diverge from class I because, although derived from cDNAs, then expressed genes, they encode unusual proteins which have not been observed until now, and display new features, such as histidine residues, along with a flexible 98–101 amino acid size, and additional computer-deduced features, such as Asn glycosylation sites. Most of the elicitin-like sequences identified within the *Pythium* (or *Pythium*-like) species fit into this class [29].

● Class II contains HAE (hyperacidic elicitin) sequences, i.e. 103–104 amino acid-long ORFs from *P. cryptogea* [38] or *P. cinnamomi* [39] which would correspond, if expressed, to highly acidic proteins, exhibiting net charges ranging from − 6 to − 10. To date, no peptides or trancripts have been observed within the corresponding species, but cDNAs that encode highly acidic peptides have been identified in *P. insolita* [F. Panabières et al., unpublished results].

● Class III contains two elicitin-encoding sequences from *P. infestans* [40], which would encode 165–170 amino acid-long peptides that consist of the conserved 98-amino acid elicitin signature, followed by an ~ 70-amino acid C-terminal domain. This region, which displays a highly biased amino acid composition (Ser, Thr and Ala residues comprise 75% of the region), could represent an O-glycosylated domain. From the combination of a putative extracellular domain (the elicitin signature) and a serine-threonine rich O-glycosylated

(or potentially glycosylated) domain, an analogy was proposed between the class III elicitins and cell surface- or cell wall-associated glycoproteins; therefore, a cellular location has been proposed for these proteins [40]. If elicitins (or elicitin domains) actually occur at two locations, as extracellular and cell wall proteins, novel hypotheses based upon dimerization events would be very attractive and would give new insights for this not so well characterized protein family.

Structural domains

Apart from these intrinsic characteristics, elicitin sequences generally revealed other features which may be involved in their biological activity, and additional domains can be evidenced from a scanning of domain databases such as PROSITE (table 1) [41]. Thus, protein kinase C-dependent phosphorylation sites were found in the carboxy-terminal region (residues 92–94) of most of the basic, highly necrotizing elicitins, whereas the great majority of α-elicitins lack them. Moreover β-cryptogein (isolated from P. cryptogea) and β-drechslerin (from the closely related species P. drechsleri) possess an additional PKC-dependent phosphorylation site, located within the Ω-loop (residues 37–39). Another domain found in elicitin sequences is a myristoylation site, also located in the carboxy-terminal part of the sequence (residues 79–84). Finally, a helix-loop-helix, dimerization domain signature was observed in all elicitin sequences, although it corresponds to a small region (residues 76–91). This domain may be

constituted by two amphipathic helices linked by a region, which would form a loop. The signature observed in elicitins corresponds to one complete amphipathic helix. Interestingly, this domain completely overlaps the antiparallel two-stranded β-sheet revealed in the structure of cryptogein. If valid, this observation would suggest that several alternative structures might occur. Dimerization of elicitins is frequently observed in SDS-polyacrylamide gel electrophoresis (PAGE) and was recently reported [35], so that the exploration of this property may be relevant while dissecting the functions of elicitins.

It has to be noted that all these features proceed from computational analyses of elicitin sequence. Thus, the occurrence of posttranslational modifications still remains to be demonstrated, the removal of signal peptide excepted. Refined tools for structural analysis of elicitins, especially within the fungus, or when translocated in planta, must then be developed.

Elicitin genes: basic bricks of a puzzling family

The high conservation of elicitins at the protein level suggests that either elicitins are young in evolutionary terms, since mutations have not altered the intrinsic nature of these proteins, or the selection pressure is high enough to maintain the cohesion of the family among the genus, whatever the species and the evolutionary events that affected the rest of the genome. So it is likely that elicitins are also conserved at the nucleotide level and share some structural features that may be typical

Table 1. Structural characteristics of the different elicitin classes.

Class	pI range	PROSITE domains	Proteins
Iα	4–5	myristyl helix-loop-helix	cactorein, cinnamomin, cryptogein, citrophthorin, INF1, megaspermin, parasiticein
Iβ	7.5–8.5	PKC phospho-site myristyl helix-loop-helix	cryptogein, drechslerin, megaspermin
I'	4.6	N-glycosylation myristyl helix-loop-helix	œdochilin
II	3.5	CK2 phospho-site myristyl helix-loop-helix	cinnamomin-ha1, cinnamomin-ha2, cryptogein-ha1, cryptogein-ha2
III	3.5–4.0	N-glycosylation cAMP phospho-site PKC phospho-site CK2 phospho-site leucine-zipper myristyl helix-loop-helix	infestin 2A, infestin 2B

of the whole family. Despite the paucity of available data (only nine genes were characterized from *P. parasitica* [42], *P. cryptogea* [38] and *P. cinammomi* [39], and three additional messenger RNAs (mRNAs) from *P. infestans* [40, 43]), common features can be defined.

Organization of elicitin genes

First, elicitins are encoded by small, multigene families. The number of genes varies from one species to another, as observed by Southern hybridizations [29, 38, 44], but does not appear to display intraspecific variation [F. Panabières et al., unpublished]. The multiplicity of elicitin genes does not totally overlap the complexity of elicitins found in the culture filtrates; thus, an elicitin isoform can be encoded by several copies, whilst some others appear to be unique. For example, β-cryptogein and parasiticein (from *P. parasitica*) are encoded by at least two identical copies [45, 46].

Four different elicitin genes were found to be clustered on a ~5-kb region within *P. cryptogea* [38] as well as within *P. cinnamomi* [39]. All the genes share the same transcriptional orientation in these clusters. A refined analysis on a genomic clone of *P. cryptogea* revealed that at least 3 kb are duplicated in the vicinity, so that at least six elicitin genes (and maybe more) constitute this cluster [45]. Southern experiments and sequence data suggest that elicitin genes occur within a single cluster in *P. cryptogea*.

Structural characteristics of the elicitin genes

The various elements that constitute the elicitin genes can been analyzed as follows.

Coding regions. Elicitin genes that have been characterized so far lack introns [29, 38, 39, 42]. However, splicing is likely to occur in *Phytophthora*, as deduced from the characterization of introns in some genes isolated from *P. parasitica* [47] and *P. infestans* [48]. Moreover, sequence analysis of the intron-exon boundaries indicates that splicing events that may occur in *Phytophthora* are similar to those of higher organisms [49].

Signal peptide. Elicitins, like other secreted proteins, are synthetized as preproteins that undergo posttranslational modifications through the removal of a signal peptide. This segment is typically 18–20 amino acids in elicitin precursors [29, 38, 40, 42, 43], and is highly conserved among all elicitins studied so far [29, 38], suggesting that elicitin precursors are probably processed and secreted following similar mechanisms.

5' untranslated regions (5' UTR). This segment of elicitin mRNA is generally short, in a 40–60-bp range. Despite its small size, this region has interesting features. First, it allows the characterization of the tran-

scription start site (TSS) of elicitin genes. It has been experimentally identified in *parA1* [42] and in *B14*, the gene encoding α-cryptogein, using the primer extension technique, then by sequence comparison on other elicitin genes [38]. This site encompasses a short (17-bp) sequence which occurs in several genes identified so far not only in *Phytophthora* [38] but also in other Oomycetes, such as *Bremia lactucae* [50]. From this, it is now considered an oomycete-specific transcription initiation consensus sequence [51]. Another feature of the 5' UTR is that cytidine residues are particularly abundant and may be derived from a microsatellite sequence that would be based upon the (CCA)n motif. This motif, which is more or less conserved, is mainly located on the 3' half moiety of the region, but may represent up to 60% of the 5' UTR. This motif has not been observed to date in other *Phytophthora* genes, and could be a signature of elicitin genes. In addition, a CAAG motif has been found to precede the initiation codon in all elicitin genes from *P. cryptogea* and *P. cinnamomi*, regardless of the class of gene. In contrast, this consensus is not found upstream of the start codon of *parA1* or *Inf1*, or other genes from *Phytophthora*. As a result, it appears that regions overlapping the start codon are particularly well conserved among some elicitin genes, and are specific to them. This region corresponds to the ribosome binding site [52]. This sequence specificity, associated with strong conservation, suggests that specific mechanisms govern the translational control of elicitin gene expression. This speculation may be enforced by sequence comparisons. As already observed with signal peptide sequences, 5' UTRs of equivalent elicitin genes are well conserved. Thus the β-elicitin mRNAs from *P. cryptogea* and *P. cinnamomi* possess 5' UTRs that are 57 and 58 bp long, respectively, which share 84% similarity. The 5' UTRs of acidic elicitins are 63 and 67 bp long, respectively; the similarity reaches 85%. In the case of the HAE genes, the similarity is not so high, and 'only' reaches ~63%. Here again, the various elicitin genes display class-specific characteristics, which may be involved in the regulation of gene expression.

3' untranslated regions. 3' UTRs are generally a rich repository of cis-acting regulators of gene expression, which contain signals determining mRNA localization and controlling polyadenylation, mRNA stability and signals controlling translation initiation [53]. In addition, they have been shown in some cases to control gene expression at the transcriptional level [54]. However, the analysis of 60 different 3' UTRs issued from 32 *Phytophthora* species revealed only few noticeable features or potential secondary structures [F. Panabières et al., unpublished results]. As a rule, the 3' UTRs are highly variable, from 103 bp (in *P. heveae*) to 197 bp (β-cryptogein). The base composition varies from

37.6% GC (in *P. megakarya*) to 58.7% GC (α-elicitin from *P. megasperma*). Consequently, proper sequence alignment is quite impossible, although some sequences can be clustered. Interestingly, these regions, although largely diverging, are strictly conserved for a given gene at the species level, as observed for various elicitin genes from *P. cryptogea* [F. Panabières et al., unpublished results] or *P. cinnamomi* (F. Panabières et al., unpublished results [39]). Nevertheless, two canonical sequences could be defined. The first one is constituted by the stop codon, which is highly conserved. Thus the UAA codon is prevalent among all the sequences analyzed (about 87%). A notable exception is *inf1*, which possesses a UGA stop codon [43]. The first nucleotide located downstream of the stop codon is generally a G, even in the case of *inf1*, generally followed by a purine-pyrimidine doublet. More precise is the consensus sequence that corresponds to the polyadenylation signal site. This canonical sequence, which is generally AATAAA in most higher eukaryotes [55], is represented here by ATGAA, located 11–25 bp upstream from the 3' end, which is itself frequently preceded by a AUAAA sequence, 11–15 bp upstream. This sequence is also encountered in other *Phytophthora* sequences, so that it may represent a signature at the generic level.

Diversity of elicitins from an evolutionary point of view

Elicitins have been shown to represent a fertile, open field for research in plant pathology and mycology, since more than 100 papers devoted to these proteins have been published within the last 15 years. However, most of them used elicitins as tools for studying plant defense responses (for review, see [28]), if not lures that could mimic the incompatible interaction. On the other hand, elicitins have been considered avirulence factors on tobacco [42, 43, 56, 57], so that *parA1* and *inf1* were described as avirulence genes cloned from *Phytophthora* spp. [58]. As a consequence, the basic, if not intrinsic, role of the elicitins has been poorly investigated. The delay observed between the first description of elicitins [14] and the recent demonstration of a biological property, if not a function for elicitins [59], is rather demonstrative. Thus several black boxes and paradoxes remain to be explored, especially on the 'fungal side'. For instance, why are elicitins so diverse yet so conserved? This point has not been taken into account in the elicitin story, and remains an enigma. Is there a correlation between the diversity of elicitin genes within a given species and some genetic, biological, physiological or pathological traits of interest for *Phytophthora* that could help in defining of an ultimate function.

Evolutionary relationships between elicitins: taxonomical consequences

The significance of the diversity of elicitin genes can been assessed at several levels. At a first level, this diversity (revealed by high performance liquid chromatography (HPLC) analysis or sequence data) can be examined as a phenotypic character, and a useful landmark of *Phytophthora* species. Hence, the extreme intraspecific conservation of elicitin sequences reflects the coherence of *Phytophthora* species, such as *P. parasitica* [46], whereas the nature and distribution of elicitins is quite variable among groupings formerly associated under the '*P. megasperma*' nomenclature [F. Panabières et al., unpublished results], and reassessed as several distinct species [60]. As another example, elicitins from *P. cryptogea* are easily distinguished from those of *P. drechsleri* [29, 38], although these two species are frequently merged or considered part of a continuous, polyphyletic complex [61, 62].

The 60 species described so far within the genus *Phytophthora* are typically classified into six groups according to the morphology of reproductive structures [63], in particular whether sporangia are papillate (groups I and II), semipapillate (groups III and IV) or nonpapillate (groups V and VI). This grouping was not 'intended to imply that this is a natural classification [64]'. Yet, this temporary classification was validated 30 years later by the ribosomal DNA (rDNA) analysis of the ITS I and ITS II [65, 66]. However, other criteria, such as the attachment of the antheridium to the oogonium (paragynous or amphigynous) that made it possible to distinguish groups I and II, III and IV, V and VI, respectively, were not supported by molecular data. In addition, the separation of semipapillate and papillate species was not possible from ITS analysis [66]. Thus sporangium morphology is a relevant basis for classification. It remains to be examined whether the diversity observed among elicitins follows the classification of species as a neutral character or whether it reflects another area of relationship within the genus.

To date, up to 30 species have been investigated for the complexity of their elicitin patterns ([28], F. Panabières et al., unpublished results), by Southern hybridization and HPLC experiments. Some species possess and express a single class of elicitins, whereas others display complex patterns [28]. The first group is mainly composed of papillate and semipapillate species, whereas nonpapillate species generally exhibit a complex pattern. As exceptions, *P. quercina*, a papillate species recently isolated from diseased oak in Europe possesses an elicitin pattern close to that of the nonpapillate *P. cryptogea*, whereas *P. gonapodyides*, which belongs to the morphological group VI, only secretes one type of elicitin [67]. A common trait between all *Phytophthora* species studied is that they all

possess genes encoding α-elicitins, which are likely to be expressed [F. Panabières et al., unpublished results]. It must be noted that the elicitins identified within the *Pythium* species also belong to the class of acidic proteins [29]. Thus α-elicitins represent the paradigm of the family. Conversely, β-elicitins or relative genes are present only in some species. They were first described in *P. cryptogea* [38, 68], *P. drechsleri* [69], *P. megasperma* [70] and *P. cinnamomi* [39, 71]. To date, β-elicitins or their genes have been identified in 17 species, among which 15 are nonpapillate species [28] [F. Panabières et al., unpublished results]. Ten among the 13 species that constitute group VI were analyzed and shown to possess β-elicitins [F. Panabières et al., unpublished results]. Thus three apparently independent characters (papilla type, ITS sequences and basic elicitins) appear to be of equal significance for classifying species.

At the amino acid level, a comparative analysis of available sequences confirms some phylogenetic data obtained from rDNA analysis and brings new information. Hence, the elicitins from group I species (i.e. cactorein, idaein, iranicin and pseudotsugaein) are tightly clustered, if not similar (fig. 3). Two other elicitins, PARA1 from *P. parasitica* and INF1 from *P. infestans* are linked to this group. Such a grouping has already been observed by rDNA analysis [66] and isozyme data [72]. Another group consists of elicitins isolated from *P. citrophthora* and *P. capsici*. Here again, the close relationship between these two species was previously noted using various criteria [66, 72, 73]. This analysis also confirms that *P. sojae* and *P. megasperma*, previously regarded as members of the *P. megasperma* species complex [74], represent distinct lineages, as indicated with rDNA analysis [65]. In contrast, some species previously clustered possess distant elicitins. Such is the case of *P. megakarya* and *P. palmivora*, both pathogenic on cocoa, and associated in molecular analyses [66, 73], or *P. citricola*, which is distant from the *P. capsici/P. citrophthora* group, on the basis of its elicitin, whereas it is closely related to them from isozyme data [75]. Finally, the clustering of the elicitins from the nonpapillate species does not fit the rDNA-based phylogeny, as basic and acidic elicitins broadly constitute distinct clades. It thus confirms that elicitins are sound tools for the classification, if not identification of species, but cannot be used in phylogenetic analyses as with ITS [29]. The comparative analysis can be extended to the signal peptides, as deduced from genomic clones [38, 39, 42] or full-length cDNAs [40, 43]. From this, it appears that signal peptides are characteristic of α- and β-elicitins, respectively, whatever the species are (fig. 4). In addition, HAE genes possess distinct signal peptides, as the elicitin-like genes from *P. infestans*. From the comparison of phylogenetic inferences deduced from

the alignment of elicitins, in their mature form, and their relative signal peptide, it is tempting to hypothesize that different selective forces act on signal peptides and mature proteins. It further supports the idea that the processing and further secretion of elicitins are important events for *Phytophthora*, and investigations in the secretion processes would be of prime value in unraveling elicitin functions.

Diversity of elicitins: biological significance?

The observation that elicitin diversity sometimes fits the *Phytophthora* phylogeny and sometimes not implies that additional correlations have to be searched with other traits that do not follow the rDNA- or papillation-based classification. However, the disconnected distribution of basic and acidic elicitins permits elimination of some candidate characteristics. Among them is obviously the antheridial attachment, as indicated above. Hence, both paragynous (group V) and amphigynous (group VI) species possess basic elicitins, and a close cluster contains elicitins from paragynous (*P. cactorum*, *P. idaei*) and amphigynous species (*P. parasitica*, *P. infestans*). Another trait is the type of sexual life cycle. *Phytophthora* species are either homothallic (self-fertile) or heterothallic (self-sterile). Here again, β-elicitins are observed in both homothallic (*P. megasperma*, *P. syringae*) and heterothallic species (*P. cryptogea*, *P. cinnamomi*). In addition, PARA1 and INF1, secreted by two heterothallic species, are close to elicitins from the homothallic species of the group I.

Phytophthora species are diverse in that some of them attack one or a few species of plants, whereas others have a very broad host range. The host specialization is not a phylogenetic criterion, and elicitin distribution is not related to the host range. For example, *P. pseudotsugae*, which is pathogenic only on Douglas fir [76], is closely related to the broad host range *P. cactorum* [66], following rDNA-based phylogeny. These two species possess and express nearly identical elicitin genes. Another example, *P. parasitica*, which is by far one of the most polyphagous species, exhibits phylogenetic affinities to *P. infestans*, which is restricted to solanaceous plants [76]. These affinities are also observed at the elicitin level. In addition, an unexpected cluster links the elicitins from low-temperature, host-specialized *P. fragariae* and the broad host range species *P. palmivora*, mainly located in tropical areas [76]. Isoform *soj2* from the host-specific *P. sojae* [77] is clustered with the β-elicitins from the broad host range species *P. cryptogea* or *P. cinnamomi*. In addition, as a consequence of the extreme intraspecific conservation of elicitin distribution, isolates of *P. parasitica* that are pathogenic and specialized on *Citrus* possess and express the same set of elicitin genes as those of broad host range isolates [V. Colas, personal communication]. Moreover, the broad
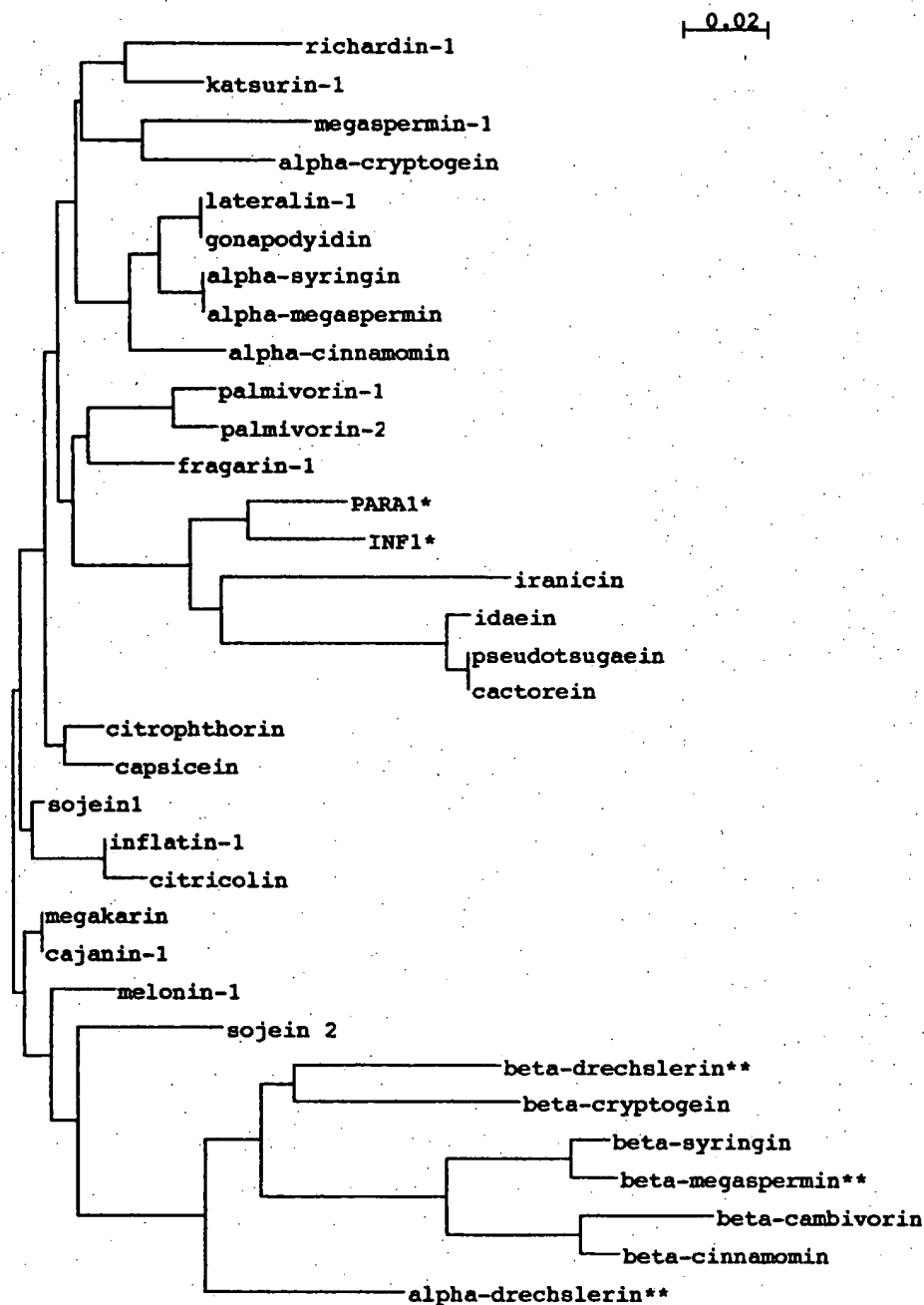
Figure 3. Phylogenetic relationships between elicitins, inferred from sequence alignment. Unless otherwise indicated, sequences were deduced from cDNA analysis. Additional sequences have been included, such as PARA1 and INF1 (*[42, 43]), α-and β-cinnamomin [39], and data obtained from protein sequencing (**[69, 70]). Trees were constructed using the neighbour-joining method based on the multiple alignment of elicitin sequences performed with the Clustal W software [165]. Confidence limits (indicated in% at the nodes) were created in a bootstrap analysis using 10,000 replicates.

host range species *P. cryptogea* exhibits an elicitin pattern similar to those displayed by the host-specialized *P. quercina* [67] or the exceptional *P. undulata*, which has not been proven to be a pathogen of any plant [29, 76].

Some species, like *P. infestans*, infect aerian parts of the plants through the development of specialized infection structures named appressoria [78, 79], but *Phytophthora* species are generally soilborne root pathogens and do not develop such structures. Moreover, *P. infestans* is classically described as typical of hemibiotrophic pathogens, which developed intimate interactions with host plants, if not coevolved with their hosts [80]. Conversely, *P. parasitica* can be considered a typical broad host range, soilborne pathogen. However, the close affinity between PARA1 and INF1 impedes such a distinction on the sole basis of elicitin sequences.

From all these comparisons, it appears that the distribution of elicitins among the *Phytophthora* genus may fit some criteria of phylogenetic relevance, such as the papilla morphology, already supported by rDNA-based classification. Hence, these characteristics may have been submitted to equivalent evolutionary controls. In contrast, elicitin diversity cannot be linked to other sexual traits or pathological behaviors, and is unlikely to be involved as a determinant of host range, if not in

pathogenesis, despite previous statements [26, 38, 43, 56, 58]. Moreover, accumulated evidence indicates that the different regions which constitute elicitin-encoding sequences are likely to be submitted to various, distinct evolutionary forces, which may be independent of other functions traditionally explored in the case of pathogenic fungi. Hence, alternative roles or functions must be proposed for elicitins, especially if we consider that this protein family is restricted to a narrow phylogenetic niche, as are the *Pythiaceae* [29, 81]. Moreover, their unique situation and their various properties described below suggest that if it occurs their role or function must be common for all the elicitins, and subsequently for all *Pythiaceae*. In this context, the most obvious unique feature of *Pythiaceae* among Oomycetes is that they are unable to synthesize sterols, but have been frequently described to require an exogenous source of β-hydroxy sterols for sporulation [76], despite debating hypotheses (M. Ponchet and P. Venard, unpublished results, [82]). The recent demonstration of a sterol-binding activity for elicitins [59, 83, 84] suggests that this track needs to be further explored. On the other hand, if elicitins share a common function, they are likely to be under the control of similar regulatory events. It is therefore worthwhile examining the regulation of elicitin gene expression.
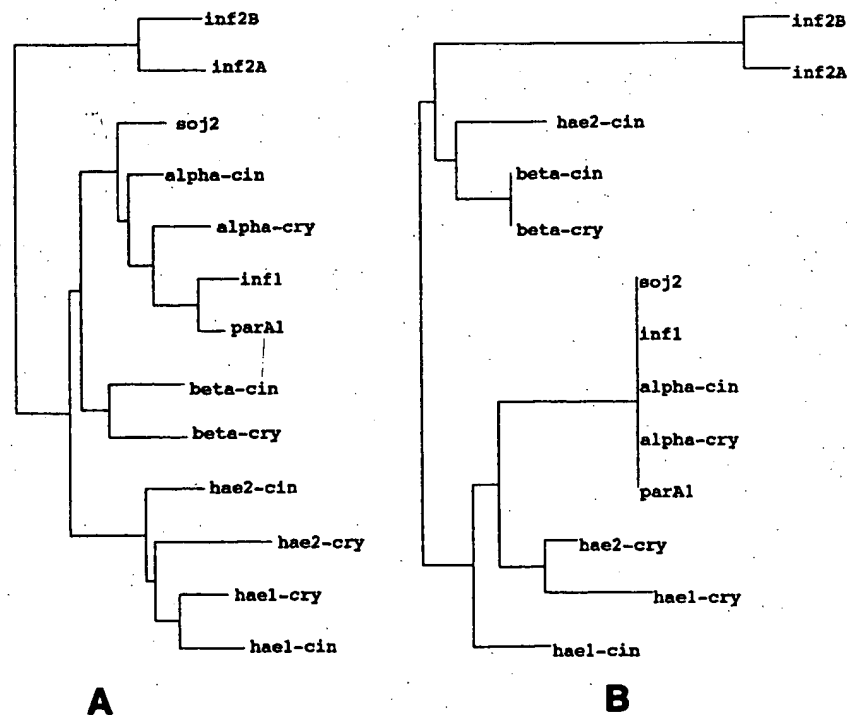

A   **B**

Figure 4. Phylogenetic inference of some elicitin genes deduced from the sequence alignment of the sequence of the mature peptides (*A*) or their corresponding signal peptides (*B*). Phylogenetic analyses were performed as described in figure 3.
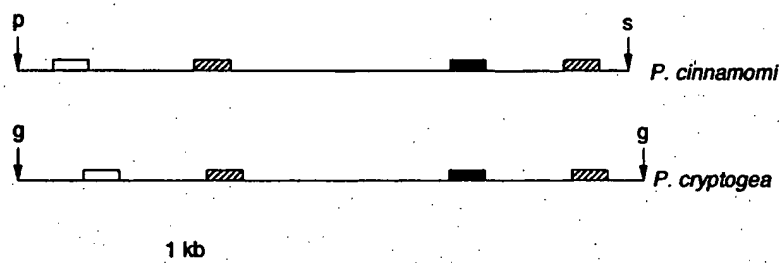
Figure 5. Schematic representation of the organization of the elicitin gene cluster, as determined in *P. cinnamomi* (upper, [39]) and in *P. cryptogea* (lower, [38]). The genes encoding β-elicitins are represented by white rectangles, and the genes encoding α-elicitins by black solid rectangles. Dashed rectangles indicate the different genes encoding hyperacidic elicitins. The limits of the clusters are indicated by restriction sites for *Bg*lII (g), *Sph*I (s) and *Pvu*I (p), as described in the original papers [38, 39].

Regulation of elicitin gene expression

Is elicitin gene expression constitutive? Elicitins have been generally described as the most abondant peptide in culture filtrates of *Phytophthora* spp. under various culture conditions [28, 44]. The tight correlation between the dosage of various isoforms of elicitins from *P. cryptogea* and their corresponding mRNAs in vitro may suggest that their expression would be constitutive [38]. However, the gene encoding the elicitin from *P. infestans*, called *Infl*, was shown to be expressed only at the mycelial stage when observed in vitro [43]. In addition, isolates usually referred to as *P. parasitica* var. *nicotianae*, which are highly pathogenic on tobacco, generally fail to secrete elicitins, though they retain a set of elicitin genes [42, 44]. Thus, the regulation of elicitin gene expression remains an open question, and the consequences of this regulation are diverse. Are all elicitin genes regulated following identical pathways, subsequently suggesting that they share a common role or function? If not, do distinct regulation pathways occur for the various classes of elicitins (acidic, basic, hyperacidic)? Basically, is elicitin gene expression constitutive or is it regulated, i.e. inducible or repressible? Some preliminary elements can be provided by structural and functional analysis of elicitin genes and their putative promoter sequences. Available data concern the elicitin genes from *P. cryptogea* and *P. cinnamomi* [38, 39] and to a lesser extent *P. parasitica* [42]. First, elicitin genes of *P. cryptogea* and *P. cinnamomi* are tandemly clustered, as indicated above. The clusters of *P. cryptogea* and *P. cinnamomi* display a similar genomic organization (fig. 5). Genes belonging to the same class (β, α or HAE) occupy corresponding positions in the two clusters, which can be easily aligned. Moreover, the regions that separate elicitin genes are of similar length in both species. A similar interspecific conservation of cluster organization is observed in the case of *Hox* genes [85], globin genes [86] and in numer-

ous multigene families whose expression is known to be developmentally regulated [87, 88].

It has been shown that elicitin genes, like other genes from *Phytophthora* and Oomycetes, share a consensus sequence that corresponds to the TSS [38, 51, 89]. However, an alignment of the relative TSSs of the various elicitin genes indicates a biased conservation between the TSSs of the α- and the β-elicitins and to a lesser extent HAE genes (fig. 6). So perhaps interspecific, conserved, factors act as class-specific regulators of elicitin genes expression. The availability of sequences deduced from genomic clones allows the identification of putative promoter regions of the various elicitin genes. Sequence analysis was performed up to 150 bp upstream of the TSS of *parA1* and in a 0.4–1.8-kb region upstream of those of elicitins from *P. cinnamomi* and *P. cryptogea*, and failed to identify TATA

| β–CRY | CTCATTCTGCAATTTGC ccagtcac |
| β–CIN | CTCATTCTGCAATTgGC tccgtcac |

α–CRY	GCCATTGTGCAATTTGC tctgtcac
α–CIN	GGCATTGTGCAATTTGC tctgtcgc
α–PAR	GCCATTGTGCAATTTGC tctcatcc

HAE–CRY1	CTCATTCCTCAATTTcC cttgccaa
HAE–CIN1	CGCACTCCACAATTTGa aatttgcc
HAE–CRY2	CTCACTCCACAATTTGC cttgccaa
HAE–CIN2	TTCATTCTGCAATTTGC tttgccga

Figure 6. Sequence alignment overlapping the TSS of elicitins. When experimentally defined, the nucleotide corresponding to the precise origin of transcription is indicated (underlined). The 'oomycete-specific transcription initiation consensus sequence' is indicated in uppercase, whereas the 5' ends of the transcripts corresponding to different elicitins are represented by lowercase.

boxes as typically occurring 30–70 bp upstream of the TSSs of RNA pol II–transcribed genes [90]. However, sequences only slightly related to TATA boxes can be found 140–200 bp upstream of the TSSs of elicitin genes from the *P. cryptogea* cluster, whereas such features do not occur at all within the *P. cinnamomi* cluster. So, elicitin genes would be expressed through the control of TATA-less promoters. Such promoters are frequently observed in genes which are prone to undergo developmental [91], tissue-specific or cell cycle-specific regulation [92]. In summary, although additional elements are necessary for further hypotheses, the combination of an interspecific, conservative clustering of elicitin classes, a biased, class-specific sequence of the TSS and the likely 'TATA-less' structure of the promoters, as well as a stage-dependent expression of *inf1* likely suggest that elicitin gene expression is not constitutive but may undergo specific regulation events.

Functional analysis of an elicitin promoter. The hypotheses presented here, concerning potential class-specific modulations of elicitin gene expression, do not contradict the notion of a coarse, more or less common, regulation pathway for elicitin genes among the *Pythiaceae*. This is strengthened by the characterization of sterol-binding properties of various elicitins [83], along with their overall high sequence conservation. Thus if elicitins share a similar role among all *Phytophthora* species, the mechanisms that promote their expression and final secretion must be highly similar. In order to test this hypothesis, a gene encoding β-cryptogein from *P. cryptogea* was used for the transformation of *P. infestans*, resulting in the transcription and translation of this gene, and the efficient secretion of the corresponding peptide [93]. Structural analysis of the transformant, called H9, indicated that the introduced sequence had remained intact and was still flanked by ~ 860 bp 5' upstream of its TSS, and then was likely to retain its own putative promoter. The amount of cryptogein expressed in H9 and in *P. cryptogea* grown under identical conditions were similar. Consequently, the cryptogein gene is flanked by a region that is likely to contain all the elements that constitute its promoter. This promoter is functional and is expressed to the same extent within the two genomic environments. On another hand, the overall amounts of elicitins are similar in H9 and in a *P. infestans* isolate which was transformed with a gene that confers antibiotic resistance. Thus it appears that due to a defined, optimal amount of elicitin production, the expression of the alien cryptogein gene led to the lowered expression of the endogenous *inf1* sequence. As a result, the phenotype of H9 during interaction with tobacco plants was similar to that observed with the highly necrotizing *P. cryptogea*, whereas no apparent symptoms of hypersensitive necroses could be detected with the control *P. infestans*

transformant. From this analysis, we can conclude that (i) the promoters of elicitin genes are functional, and may be regulated by identical factors in various species, (ii) the overall production of elicitins is constant within a given isolate, whatever the nature of the elicitin and (iii) elicitins from different classes may be substituted without any apparent modifications for the fungus (except the phenotypic behaviour on tobacco), and therefore may play a common role within *Phytophthora* [93].

Elicitin gene expression in plant-*Phytophthora* interactions

Soon after their characterization, elicitins were compared with other small, cysteine-rich proteins that would play a role in plant-fungi interactions, such as avirulence gene products and hydrophobins [94]. The first observations indicated that isolates which produced elicitins were nonpathogenic on tobacco, whereas the strains virulent on tobacco did not produced parasiticein [18]. As elicitins are specifically active on *Nicotiana* spp. [18], it was likely that the absence of elicitin secretion was a basis for pathogenicity to tobacco, and elicitins, from their ability to induce both HR and SAR on tobacco, would therefore be considered virulence factors. Unlike other plant-fungus interactions which involve the recognition of race-specific elicitors [95], elicitins, if acting as avirulence factors, would behave like species-specific elicitors. However, virulent, nonproducing isolates were shown to retain elicitin genes [42, 44], which organization, similar to that occurring in nonpathogenic strains, impedes distinction between virulent and avirulent strains [44, 46]. Later, some *P. parasitica* isolates from Australia were shown to produce parasiticein, but were still virulent on tobacco, although to a lesser extent than nonproducing strains [20]. Analysis of a worldwide collection of *P. parasitica* isolates, mostly from tobacco growing areas, revealed the occurrence in South America as well as in Africa of some strains that were highly virulent on tobacco but still producing parasiticein, at least in vitro [96]. If elicitins actually act as avirulence factors on tobacco, the diverse situations imply that the development of virulence would be the outcome of several distinct mechanisms among *P. parasitica*, such as potential repression of elicitin gene expression among virulent, producing isolates. The expression of elicitin genes was thus evaluated in various types of interactions.

Incompatible interactions have been poorly studied because the lack of fungal development is a technical limitation for accurate transcriptional analysis. Nevertheless, elicitin production has been investigated by immunological detection of cryptogein in the leaves and stems of decapitated tobacco plants inoculated with a

mycelial plug of *P. cryptogea* [21]. However, it was not possible to determine whether it reflected actual transcription of the elicitin gene or the release of a previously synthesized peptide. The role of elicitins in incompatible interactions was also studied using an indirect approach. Indeed, histological analyses indicated that *P. infestans* induced symptoms suggesting an HR response when inoculated on *N. benthamiana*, a solanaceous species which is known to be deficient in displaying defense response to viral pathogens [56]. When inoculated by antisense transformants of *P. infestans* which no longer produced INF1, this particular *Nicotiana* species in some cases exhibited symptoms of disease, related to those that occur in the compatible interaction of *P. infestans* with potato. From these experiments, the authors concluded that 'the recognition of elicitin is a major determinant of the resistance response of *N. benthamiana* to *P. infestans*', and that 'elicitins are avirulence factors that condition resistance at the species level' [56]. It should be noted that *P. infestans* is restricted to solanaceous plants and mainly pathogenic on potato and tomato [76]. Thus we may speculate that *P. infestans* possesses additional virulence factors that are balanced by elicitins in interaction with atypical *N. benthamiana*, and opened out in INF1-deficient transformants. Whatever the relevance of these results, they do not reflect the wide majority of interactions between *P. infestans* and other solanaceous plants, and therefore *Phytopthora* spp. and the diversity of host plants. Hence, the phenotype of INF1-deficient strains is not modified when inoculated on other *Nicotiana* species, or on potato [56]. In the same context, *P. infestans* transformants that expressed β-cryptogein in addition to INF1 were not altered in their virulence against potato or tomato [P. Birch, personal communication]. Nevertheless, the results obtained in the INF1-deficient strains-*N. benthamiana* interaction are relevant enough to offer a promising innovative field of research on the role of elicitins as determinants of host resistance.

Immunological methods have also been used to analyze elicitin production during various compatible interactions [23]. The correlation between the observation of symptoms and the detection of elicitins was rather variable, but clearly demonstrated in the cases of *P. parasitica*-tomato, *P. capsici*-tomato and *P. capsici*-pepper. There, elicitins could be detected 1 or 2 days following the inoculation of plants. Elicitin production was analyzed at the transcriptional level in the *P. infestans*-potato interaction [43]. In this system, expression of *inf1* did not follow the increase of fungal biomass during infection but only occurred in the late phase of fungal invasion, then reached a maximal level and decreased after 5 days following inoculation. This step corresponds to the transition between the hyphal growth and the phase of extensive sporulation. Conversely, expression of actin genes, presented to be constitutive, follows the development of invasion. Moreover, on the basis of the comparison of overall transcription of *inf1* in planta and in vitro, the authors concluded that expression of elicitin genes is downregulated during infection of potato [43]. As indicated below, *P. infestans* is a hemibiotroph, airborne pathogen, whereas most *Phytophthora* species are root, soilborne pathogens. However, several species are able to colonize aboveground tissues following rain splashings [76]. Therefore, such species must be analyzed for the time course of elicitin gene expression during infection in order to determine whether *P. infestans* offers a particular situation or whether it reflects a general regulation pathway for expression of elicitins. Actually, the response of a plant to elicitins, as exemplified by *N. tabacum*, represents an exception in the plant kingdom [97, 98]. It is difficult to consider downregulation of elicitins as an adaptation of *Phytophthora* spp. to evade plant defense responses that could have been triggered by elicitins; thus downregulation of elicitin gene expression may find its source in the events leading to various changes in cell types during infection, rather than direct interaction with the host plant. Finally, it remains to be seen whether downregulation occurs during infection of a plant typically responsive to elicitins, such as tobacco, by virulent strains of *P. parasitica*, which were shown to produce elicitin in vitro [20, 96], or whether specific virulence factors overcome the plant defense responses.

From these observations it may be concluded that elicitins play a special role in tobacco-*Phytophthora* interactions. This role cannot be restricted to a gene-for-gene model, since various situations are encountered, especially within solanaceous plants [20, 57, 96, 98]. Considering that the plant response (reactivity?) to elicitins is an exception in the huge diversity of plant families that are able to be infected by *Phytophthora*, the expression of elicitins during compatible as well as incompatible interactions must be further analyzed. Thus additional determinants involved in the wide diversity of interactions between plants and *Phytophthora* spp. remain to be identified.

A first approach of the biological properties of elicitins

Physiological and biochemical effects induced in plants leading to necrosis and SAR development

Cryptogein application on the petiole of excised tobacco leaves induces necroses that are correlated with histological alterations such as rapid chloroplast breakdown and the collapse of cells leading to disorganization of the parenchyma tissue [99]. In addition, treated leaves produce ethylene and accumulate phytoalexins
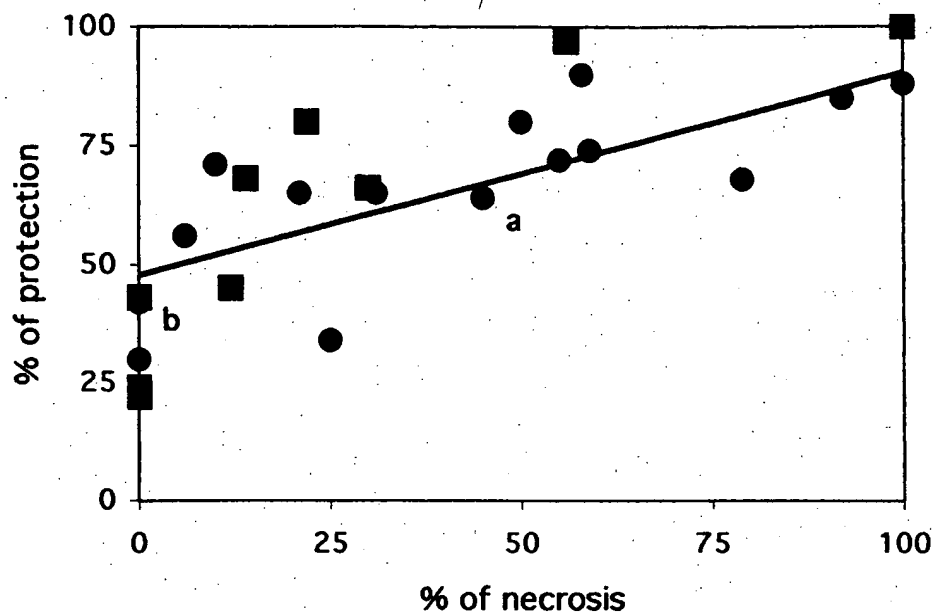
Figure 7. Relationship between necrosis and protection levels induced by mutated cryptogeins on tobacco plants. Each point represents a protein with a single or a multiple mutation. Circles and squares correspond to mutation on Lys or Tyr, respectively. 'a' represents Lys13Val and 'b' replacement of five Lys by polar and neutral amino acids.

such as capsidiol [99]. Elicitin application on the stem of decapitated plants is followed by rapid translocation of the protein in the plant [21, 22] and triggers necrosis development only when β-elicitins have been used [17]. Tobacco plants subsequently become resistant to further inoculation by pathogens. This protection depends on a complex signaling network, and its level results from the equilibrium between the intensity of plant defense and the rate of pathogen invasion. Better results are obtained when the pathogen inoculum is well quantified, as in *Phytophthora* zoospore infiltrations, than in direct contact of wounded tissues with mycelium! Elicitin-treated tobacco plants that express the bacterial *nahG* gene coding for salicylate hydroxylase do not exhibit SAR, but still respond to treatment with β-elicitins by intense leaf necrosis. Salicylic acid is clearly involved in SAR and in disease resistance to *Phytophthora*, but these results indicate that it does not mediate the hypersensitive-like necrosis response [100]. Moreover, the establishment of SAR seems to depend on the appearence of extracellular RNase activity, especially upon challenge infection [101], in the same way as in tobacco plants induced to SAR with tobacco mosaic virus [102]. This plant response could be fundamental in SAR, since RNA molecules may carry long-distance signals in plants [103]. In addition, active oxygen species (AOS) mediate a systemic

signal network, and H_2O_2 orchestrates the plant hypersensitive disease resistance induction [104, 105]. In that way, two distinct sources (intra- and extracellular) of AOS in tobacco plants treated by cryptogein have been reported, and correlated with later cell death [106], although these cell responses could be associated with lipid peroxidation [107]. Cryptogein induces lipid peroxidation in tobacco leaves, evaluated by the accumulation of thiobarbituric acid-reactive substances as well as by high-temperature thermoluminescence emission, both indicating a progressive destabilization of the thylakoid membranes [108]. In addition, lipid peroxidation is closely correlated with the appearence of necrosis [109]. It has been recently demonstrated that the production of fatty acid hydroperoxides depends only on lipoxygenase activities [110]. Finally, expression of defense genes has been studied. Elicitins trigger the coordinate accumulation of transcripts from nine genes which have been previously shown to be expressed during establishment of SAR, β-elicitins like cryptogein inducing higher response than α-ones, like capsicein. These SAR genes are expressed locally corresponding to necrosis formation, and systemically during induction of resistance [111]. Finally, elicitins were also shown to induce a new SAR gene, encoding a β-subunit of proteasome [L. Suty et al., unpublished data].

Are necroses and protection related?

Although all the elicitins put on the stem of decapitated tobacco plants induce a SAR, only β-elicitin treatment leads to the development of leaf restraint necroses [17]. Thus necrosis does not seem to be essential for the establishment of SAR. This observation has been confirmed by site-directed mutagenesis experiments. Systematic replacement of Lys or of Tyr clearly shows that necroses are not required for protection, but strongly enhance the protection level (fig. 7). The necrotic activity of engineered proteins produced in a bacterial PT_{7-7} heterologous system and mutated on the six lysines of cryptogein, with cumulative permutations of these amino acids to uncharged residues, clearly demonstrated that (i) all the lysines and not only K13 are important to explain the toxicity of β-elicitins (fig. 7, spot a) and (ii) four to five lysines must be exchanged to obtain a typical α-elicitin as capsicein in terms of necrosis and protection level (fig. 7, spot b) [I. Penot et al., unpublished data]. The single mutant K13V still remained strongly necrogenic, so that the reported results showing that this mutation gave a capsicein-like protein are irrelevant [112]. In fact, these authors assayed the necrotic properties of elicitins by foliar infiltration, a technique inappropriate to measure this activity. All the β- and α-elicitins induce necroses after infiltration with the same subnecrotic threshold concentration ranging from 10 to 20 nM ([97], P. Bonnet unpublished results). Moreover, this mode of application is unable to lead to SAR [97]. As a final demonstration, a gene encoding the α-cryptogein B14 [38] was mutated to give V13K and T94K proteins, overproduced in the same system as described above. These proteins were found poorly and highly necrogenic, respectively [A. Marais, unpublished data]. It is obvious that the 'toxicity' of β-elicitins could not be explained by highlighting single residues, but probably results from complex modifications within the structure of the molecule that lead to different behavior in planta rather than an oversimple protein-protein or protein-ligand complex involving unique sites. The net charge and tyrosine exchanges, which will be discussed further, are part of this multicomponent determinism. In addition, considerations of the importance of residues located in positions 2 (Ala), 13 (Lys), 44 (Thr) and 94 (Lys) resulting from sequence comparison [113, 114] must be used with caution, as these locations could result from a biological shift during evolutive processes. An interesting approach in mapping sites with synthetic peptides deduced from the sequences of capsicein and cryptogein was reported [115]. The experiments were carried out with saturated peptide solution reaching 1 mM with the foliar infiltration method discussed above. In these conditions, 10^4–10^5 higher concentrations of peptides were necessary to mimic the elicitin effect, and when these peptides were infiltrated at lower concentra-

tions, it resulted in a total loss of necrotic activity. Although such differences in mapping strategies were described as usual, the presented results need additional studies to become fully convincing, all the more because they do not clearly show how the different elicitin structural components could act to explain the signaling of the entire protein.

Responses of tobacco cells to elicitin treatment

When tobacco cells are treated with cryptogein, their growth is affected, and at 100–200 nM this treatment is lethal [116]. When added at sublethal doses, cryptogein elicits a rapid (few minutes) and strong increase in pH and conductivity of the extracellular medium, followed by cytosolic acidification, without affecting the integrity of the plasma membrane [116–118]. These changes are accompanied by a transient production of AOS, like H_2O_2 [109, 117, 119]. Capsicein requires 10-fold higher concentrations than those of cryptogein to induce similar AOS levels [109]. Delayed cell responses were ethylene production (120 min) [120] and, 24–48 h after treatment, induction of lipoxygenase and of proteinase inhibitor activities [119], and phytoalexin accumulation (capsidiol, phytuberin, phytuberol) [120]. This phytoalexin production depends neither on the presence nor on the intensity of the oxidative burst [109]. During the same period of time, changes in total cell lipids have been reported [121]. The most striking changes are an increase in acylated steryl glycosides and steryl esters levels, resulting in part from the glycosylation and/or esterification of free sterols, and in the other part from transient neosynthesis and an increase in the synthesis rate of phosphatidylethanolamine [121].

Cryptogein-treated tobacco cells were also used to describe the early changes in gene expression. The accumulation of mRNAs encoding several known plant proteins was examined by Northern and slot blot hybridizations. The results indicate (i) a significant transitory accumulation of mRNA encoding plasma membrane H^+-ATPase, (ii) a fast and strong accumulation of mRNA encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase and (iii) a slow accumulation of mRNA encoding phenylalanine ammonia lyase and pathogenesis-related protein PRb1 [122]. Differential display of mRNA was used to isolate partial length cDNAs corresponding to genes differentially expressed early during elicitation of tobacco cells with cryptogein. These cDNAs were cloned and sequenced. The first hour of elicitation showed (i) a high accumulation of mRNAs hybridizable with cDNAs having sequence homologies with phenylalanine ammonia-lyase, Ca^{2+}-ATPase and lipoxygenase encoding genes, and (ii) a decrease of mRNA hybridizable with one cDNA having

sequence homology with another LOX-encoding gene. Five other differentially displayed cDNAs showed no significant homologies with known genes [123]. A combination of mRNA differential display (DDRT-PCR) and 5'-rapid amplification of cDNA ends (5'-RACE) allowed the isolation of full-length cDNAs corresponding to genes activated within 60 min. Cloning and sequencing two cDNAs led to the identification of open reading frames (ORFs) showing significant homologies with the coding sequence of β-type proteasome subunit and of a transformer-2-like serine/arginine-rich (SR) ribonucleoprotein [124]. The accumulation kinetics of mRNAs indicated transcriptional activation of the corresponding genes not only in cells but also in tobacco plants treated with cryptogein.

Proteasomes are multicatalytic complexes which catalyze the degradation of many rate-limiting enzymes, transcriptional regulators, critical regulatory proteins and highly abnormal proteins. They are involved in plant responses to environmental stresses (cold or high temperature, abscisic acid treatment, drought or salt stress), in cell death, in senescence or wounding processes. Whether the proteasome complex could play a role in the induction of oxidative burst, of cell death or of defense reactions triggered by cryptogein still remains to be studied. Using gene walking by PCR, the 5' flanking region of the β-type proteasome subunit has

been cloned and sequenced. Sequence analysis in the PLACE data bank allowed the characterization of regulatory sequences, especially mybcore and mybst1 boxes, that could be responsible for regulation by salicylic acid. Effectively, upregulation of the β-type proteasome subunit by salicylic acid in tobacco cells has been observed [L. Suty et al., unpublished data]. This upregulation was confirmed using nahG transgenic plants. H_2O_2 was also shown to upregulate the β-type proteasome subunit, and altogether these results suggest that this β-type proteasome subunit is a new marker of SAR [L. Suty et al., unpublished data].

SR proteins comprise a family of evolutionarily conserved pre-mRNA splicing factors. Transformer-2 like proteins play an important role in the alternative splicing of pre-mRNA, and SR proteins are among the first components that interact with pre-mRNA. Very little is known about SR protein functions in plants, but in comparison to those present in animals, the relative amount of each SR protein contributes to the regulation of gene expression. Identification of the target pre-mRNA could be useful to evaluate the importance of such SR proteins in elicitin signaling.

All the responses described above were likely to depend on elicitin recognition by specific high-affinity binding sites [116] and by protein phosphorylation events [125].
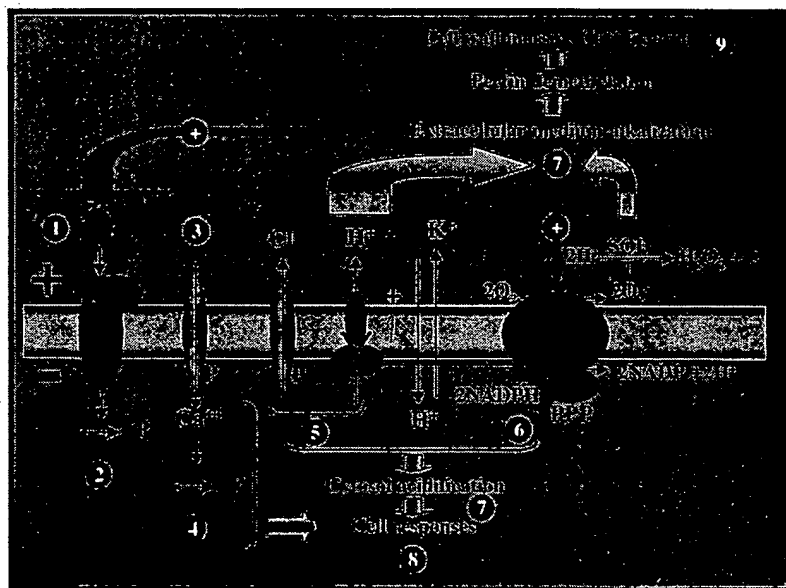

Figure 8. Elicitin signaling pathways (nine steps). Elicitin is represented by the green ellipse. From the left, the different plasmalemma proteins involved are the putative receptor (two subunits, a 160-kDa and a 50-kDa protein), a calcium channel, a chloride channel, H^+-ATPase (inhibited) and NADPH oxidase. The signs + and − indicate the transmembrane potential. The protein phosphorylation steps are indicated by the blue 'P'. Orange arrows show the systems which create the changes in pH. The blue arrows indicate the positive feedback effects of the extracellular medium alkalization, and the numbers 1-9 indicate the events in their chronological order.

Signaling pathways involved in elicitin cell responses

A specific binding of elicitin to high-affinity sites was first described at the cell level [116]. Further experiments showed that the cryptogein binding sites were located on the plasma membranes, which were purified from cell suspensions or tobacco plants [126]. The binding is saturable, reversible, specific with an apparent K_d of 2 nM (well correlated with concentrations required for biological activities in vivo), and with a very low number of sites (about 100–200 fmol/mg plasmalemma proteins), suggesting that these sites could be the biological receptors for elicitins [126] (fig. 8). Early tobacco cell responses (changes in extracellular pH and in ionic fluxes, AOS production) have been used to investigate the possible desensitization of cells by successive cryptogein treatments. After a first treatment, tobacco cells still respond weakly to a second application of cryptogein or of other elicitors such as oligogalacturonides, suggesting that elicitins induce a desensitization process which corresponds to some forms of heterologous desensitization. It indicates that exposing tobacco cells to elicitins attenuates the response due to other signals operating through distinct receptors [127].

These receptors are postulated to be glycoproteins, since plasma membranes treated with proteases and N-glycosidase F are not able to bind cryptogein [128]. The molecular mass of the elicitin receptors has been tentatively approached by cross-linking experiments that indicate two possible complexes with molecular masses of 172 ± 15 kDa and 60 ± 4 kDa, respectively, and thus the molecular mass of the cross-linked glycoproteins would be about 160 and 50 kDa [128]. This is in good accordance with the functional molecular mass of the cryptogein binding sites (193 ± 9 kDa) determined by radiation-inactivation experiments [128]. Finally, all the elicitins tested are able to bind to the same sites (with a similar affinity), suggesting that elicitins are recognized by the same receptors, although they induce differential cell and plant responses [129]. These apparently contradictory observations remain to be explained and will be discussed further.

The earliest event of the elicitin signal transduction pathway is a protein phosphorylation/dephosphorylation cascade, since all biological effects were blocked by protein kinase inhibitors, such as staurosporine or K_{252a} [118, 125] (fig. 8). This signaling involves SIP [130] and MAP kinases [131], probably at multiple steps of the signaling pathway. It leads to a huge Ca^{2+} uptake [132], since this cation reaches an apparent intracellular concentration of 200 μM after 30 min, which could be responsible for the high cryptogein toxicity [128, 129, 133]. Since EGTA, which chelates extracellular calcium, or lanthanum, which blocks calcium entry, suppresses the downstream responses, it is obvious that the calcium

entry triggers the other cryptogein-induced responses; however, the calcium amounts involved in the signal transduction need to be precisely reevaluated.

First of all, calcium uptake is not transient, and calcium accumulation is detected only 5 min after elicitin treatment and increases during the following 90 min [132]. However, changes in extracellular pH or in AOS production are observed almost immediately after elicitin addition [116, 129]. Thus it must be concluded that the high calcium concentrations observed in these experiments do not correspond to a signal transduction phenomenon and that the use of $^{45}Ca^{2+}$ is not relevant for this purpose. On the contrary, using Ca^{2+}-specific electrodes, a rapid and transient calcium uptake (restoration of the original level over 2 min), involving very low concentrations, has been reported in radish protoplasts treated with elicitins [134]. Finally, we recently observed that cryptogein induces a rapid and strong demethylation of cell wall pectins, which could result from the activation of apoplastic pectin-esterase activity via the alkalization of the extracellular medium. Electronic microscopy observations of these cryptogein-treated tobacco cells reveal that calcium probes are mainly located in the cell wall and that Ca^{2+} ions are associated with demethylated pectins [F. Kieffer et al., unpublished data] (fig. 8). These results explain the dual role played by calcium during the elicitation of tobacco cells by elicitins: (i) a strong second messenger with weak and transient uptake in the inner cell, and (ii) formation of a calcium-pectate gel leading to the reinforcement of the cell walls (fig. 8).

Depending on the calcium signaling, other ions fluxes are also modified. Cryptogein induces a K^+ efflux (probably associated with a proton influx) [125] and an efflux of Cl^- [135], this later triggering a large plasma membrane depolarization from -153 ± 15mV to -36 ± 21 mV [118]. This depolarization occurs in less than 1 min, after a lag period of about 5 min [135]. The plasma membrane depolarization could be the result of different additional causes: (i) the electron transfer through the plasma membrane, mediated by an NADPH oxidase (see AOS production), (ii) the Cl^- efflux and, above all, (iii) inhibition of the plasma membrane H^+-ATPase. This later evidence is supported by indirect observations. For example, plasma membrane depolarization and cytosolic acidification should activate the H^+-ATPase, leading to a rapid decrease in the intracellular ATP pool, which is not observed [118]. This is also supported by cryptogein effect reversion with fusicoccin, a well-known activator of H^+-ATPase [116, 117], according to similar observations reported for tomato cells treated with systemin [136]. At the same time, a strong and rapid alkalization

of the extracellular medium and a concomitant acidifi-
cation of the cytosol are observed [116–118]. A few
minutes later, a transient oxidative burst is noticed [109,
117]. The nature of AOS has been investigated. Cryp-
togein elicits an extracellular production of $O_2^{\cdot-}$ on
tobacco cells which is then dismutated in H_2O_2 by
extracellular superoxide dismutases [C. Rustérucci et
al., unpublished data]. The extracellular production of
$O_2^{\cdot-}$ results from the activation of an NADPH oxidase
[118] which seems regulated by a small G protein such
as Rac2, in a manner different from that of neutrophils
[137]. In order to describe the molecular composition of
the plant NADPH oxidase complex and to assess its
regulatory mechanisms, we have developed an approach
using a double hybrid method, first with the gp91
subunit we have recently cloned, and second with the
neutrophil Rho-GDI factor which interacts with the
regulator proteins Rac, as bait proteins [T. Elmayan et
al. and F. Plas-Simon et al., unpublished data].
Another interesting question concerns the origin of the
changes in extracellular and cytosolic pH reported
[116–118]. It was suggested that these pH changes re-
sult from superoxide dismutase activity [118, 128, 133,
138]. In that case, inhibition of the production of $O_2^{\cdot-}$
using diphenyleneiodonium (DPI), which blocks the
activity of the NADPH oxidase, or Tiron, which scav-
enges $O_2^{\cdot-}$, should restrict these changes in pH. But DPI
and Tiron abolished AOS production without any effect
on extracellular alkalization [117] according to other
reported results, whatever the plant cell/elicitor interac-
tion studied [139]. However, a precise observation of
extracellular alkalization during the time course of the
oxidative burst proves that the contribution of the su-
peroxide anion dismutation to pH changes is very low
(about 3% at its maximum level), whereas addition of
exogenous superoxide dismutase only increases this re-
sponse about 6% [Blein et al., unpublished data]. On the
other hand, we previously reported different arguments
leading to the conclusion that the depolarization of the
plasma membrane mainly results from the H^+-ATPase
inhibition [116–118, 128, 138]. Thus, it is obvious that
the changes in pH mainly result from plasma membrane
H^+-ATPase inhibition. Furthermore, changes in pH
have been shown to modulate the intensity of AOS
production by elicited cells, and the possible regulation
of the NADPH oxidase activity of plant cells by modifi-
cations of pH has been proposed [117]. In this way,
cytoplasmic acidification has been reported to be in-
volved in the complex network of cell signaling leading
to defense gene activation in tobacco [140], rice [141]
and Californian poppy cells [142]. A hypothetical sig-
naling scheme which summarizes the pathways involved
in the early responses of tobacco cells treated with
elicitin is proposed (fig. 8).

Agronomic interest

Durability and effectiveness of elicitin-induced SAR against plant pathogens

The SAR induced by three proteins (cryptogein and two
α-elicitins, capsicein and parasiticein) was reported to
be quite efficient toward several P. parasitica aggressive
strains on tobacco. High protection was obtained for at
least 2 weeks after elicitin treatment [98]. Moreover, this
resistance was not organ-specific, since it occurred in
stems, leaves and roots, whatever the locus of elicitin
application. Thus induced resistance triggered by these
proteins was not transient and could be used in plant
protection strategies. In addition, this SAR was demon-
strated to be effective against other tobacco phyto-
pathogenic fungi: Sclerotinia sclerotium, Botrytis
cinerea, Rhizoctonia solani, Erysiphe cichoracearum and
Peronospora tabacina [98, 143]. Concerning the
polyphagic and highly pathogenic S. sclerotium, R.
solani and B. cinerea, protection was observed on both
stem and leaves. Some preliminary experiments showed
that elicitins were also able to induce unambiguous
resistance against the phytopathogenic bacteria Pseu-
domonas cichorii and Erwinia chrysanthemi in a tobacco
stem challenge. Elicitins are therefore powerful elicitors
of long term and aspecific protection of tobacco plants
toward a wide variety of pathogens.

Plant specificity in elicitin-induced HR and SAR

The range of botanical species able to react in exhibiting
HR and (or) SAR after elicitin application was thor-
oughly investigated. HR and SAR were readily induced
in all the species belonging to the three sections of
Nicotiana genus. However, the intensity of induced res-
ponses was both cultivar- and species-dependent. In
contrast, none of the other Solanaceae tested developed
necrotic symptoms after elicitin infiltration or applica-
tion on the petiole of detached leaves. Experiments
achieved on bell pepper, tomato and Petunia hybrida
with different amounts of elicitins suggested that neither
HR nor SAR against P. parasitica could be put forward
[97, 98]. Nevertheless, among plants belonging to more
than 15 botanical families, including monocots and
dicots, only some members of Brassicaceae were found
to develop necroses in a cultivar-specific manner: some
cultivars of Raphanus sativus and rape (Brassica napus)
responded by foliar necrosis after elicitin treatment,
often in a dose-dependent symptom intensity. The
symptoms on detached leaves varied from yellowing at
low elicitin quantities (< 0.1 nmol per leaf) to total leaf
water soaking then turning brown with black punctua-
tions at higher amounts (> 1 nmol per leaf) (Bonnet,
unpublished data, [134]). There was a real differential
response to elicitin among radish and rape cultivars

Table 2. ^{125}I-cryptogein binding characteristics to plant plasma membranes [J.-P. Blein et al., unpublished data].

	N fmol/mg protein	K_d nM
	reactive plants	
N. tabacum	101 ± 7	8.8 ± 1.9
B. napus var:		
yudal	190 ± 15	5.7 ± 0.2
liberator	898 ± 107	13.5 ± 2.4
lirabon	251 ± 48	10.3 ± 2.1
cobra	292 ± 47	7.7 ± 1.4
	unreactive plants	
L. esculentum	148 ± 17	7.3 ± 1.3
A. thaliana	210 ± 23	10.2 ± 1.4
A. pseudoplatanus	203 ± 15	6.1 ± 1.5
B. napus var:		
darmor nain	100 ± 3	3.1 ± 0.2
jet 9	845 ± 18	22.5 ± 1.2
bolko	448 ± 29	4.0 ± 1.7
shogun	1012 ± 275	5.3 ± 1.7

which was never observed in *Nicotiana* spp. In addition, it was demonstrated that reactive radish varieties, when treated with elicitins, became resistant to the phytopathogenic bacteria *Xanthomonas campestris* pv *armoraciae* [97]. Some other cruciferous species were also reported to develop HR, with the exception of *Arabidopsis thaliana* ecotypes. A genetic approach of reactivity using rape cultivars is in progress in order to clarify the level of complexity of the mechanisms governing elicitin recognition and signaling. The results obtained on Brassicaceae, showing degrees in symptom severity ranging from null type to complete wilting and necrosis with intermediate behavior (yellowing or senescence), suggest that a continuum could exist in reactivity to elicitin. All the experiments achieved on different plants of various botanical origins were carried out with the attempt to describe HR. Whether this reaction is an obligatory mechanism in the general elicitin mode of action remains unknown. Thus it will be necessary to reassess plant reactivity not only with symptom description but also at the molecular level to evaluate stimulation of plant defense and stress pathways. However, the elicitin receptor, until now characterized as high-affinity binding sites, is present on all the plant plasma membranes assayed (table 2) and cannot be considered to be the specific support of plant reactivity; the cell wall has been proposed to play a role [83] (fig. 9). Elicitin effects on reactive and nonreactive plant cells (tobacco and tomato, respectively) have been compared. This plant cell wall/elicitin interaction is slight in tobacco and leads to an equilibrated distribution of elicitins between cells and their extracellular medium, whereas it is very strong in tomato cells. In the latter case, the extracellular applied elicitin rapidly becomes almost undetectable.

Since elicitins could be desorbed from cell walls by salt buffers, these proteins are probably trapped in these structures through ionic bonds. The tomato cell walls behave as a filter which prevents elicitins from accessing the plasma membranes. However, high elicitin concentrations are able to saturate this barrier and trigger tomato cell responses, evidencing at least that the receptors involved in elicitin signaling pathways are functional in tomato. An approach showing that the cell wall components are involved in plant reactivity is being developed [M. L. Milat et al., unpublished data].

Biotechnological implications

The ability of elicitins to trigger plant protection toward phytopathogenic microorganisms could be used by introducing artificial resistance in plants of agronomic interest. But the high toxicity of such proteins combined with their apparent plant specificity (see above) could be limiting factors; however, the introduction of elicitin genes in reactive tobacco was achieved following two strategies. The first one, using uncontrolled expression of a synthetic gene encoding cryptogein under the strong 35S promoter led surprisingly to viable transgenic plants in both homo- and hemizygous states [144]. This could be due to the intracellular localization of the protein, which consequently was unable to interact with the outside specific site located on its plasmalemma putative receptor. Inoculation of transformed lines with *P. parasitica* aggressive strains led to a low level of resistance compared with the control. This resistance was not clear, since the inoculated plants were almost ruined by the pathogen, and the protection was estimated as the number of leaves on axillary shoots emerging from the low, still living stump of stems.

The second strategy [145] was to control foreign gene expression and to address the protein to the apoplastic space, which allowed the elicitin to bind to its putative receptor located on the outer plasma membrane [126]. Controlling gene expression is a guarantee for limiting cell death in the appropriate area where plant and pathogen interact. A promoter with a weak constitutive expression threshold but strongly inducible by pathogen attack was chosen to govern the expression of the natural gene encoding cryptogein [38]. The signal peptide of the extracellular PR1a was added to the construction between the promoter and the cryptogein gene to allow protein secretion in the apoplast. Transformed lines were obtained and screened for their ability to restrain *P. parasitica* spreading. After backcrosses, stabilized F_2 lines were used to evaluate their pathogenic phenotype. These transgenic lines reacted to zoospore foliar infiltration in an HR manner and were shown to be highly resistant. This resistance appeared in any organ of the plant and was efficient against several pathogens, including fungi, viruses and nematodes.

The biotechnological use of elicitor of resistance was improved. In the particular case of elicitins, a general use for crop protection still remains hazardous, since the mechanisms leading to reactivity and SAR or LAR (local acquired resistance) set up have to be elucidated in most plants of agronomic interest.

What is the elicitin biological function?

Some phytopathogenic fungi within *Phytophthora* species are unable to synthesize sterols and therefore must pick them up from the membranes of their host plant, using an unknown mechanism. These pseudo-fungi secrete elicitins which are small hydrophilic cysteine-rich proteins, harboring a sterol carrier activity.

Sterol carrier activity of elicitins

All these proteins interact with dehydroergosterol (DHE) in the same way, but with some time-dependent differences [59, 83]. Elicitins have one binding site with

a similar strong affinity for DHE. Using a nonsteroid hydrophobic fluorescent probe, it was shown that phytosterols are able to similarly bind to elicitins. Moreover, elicitins catalyze sterol transfer between phospholipidic artificial membranes [59, 83]. In addition, these polypeptides are also able to trap sterols from biological membranes (plant cell suspensions or purified plasma membranes) and to transfer DHE from liposomes to isolated plasmalemma vesicles [84]. These results afford the first evidence for a molecular activity of elicitins, which appears to be an extracellular sterol carrier function. This property should contribute to an understanding of the molecular mechanism involved in sterol uptake by *Phytophthora*. It opens new perspectives concerning the role of such proteins in plant-microorganism interactions.

The 3D structure of a cryptogein-ergosterol complex

Recently, the 3D structure of a K13H engineered cryptogein containing an ergosterol molecule in its hydro-
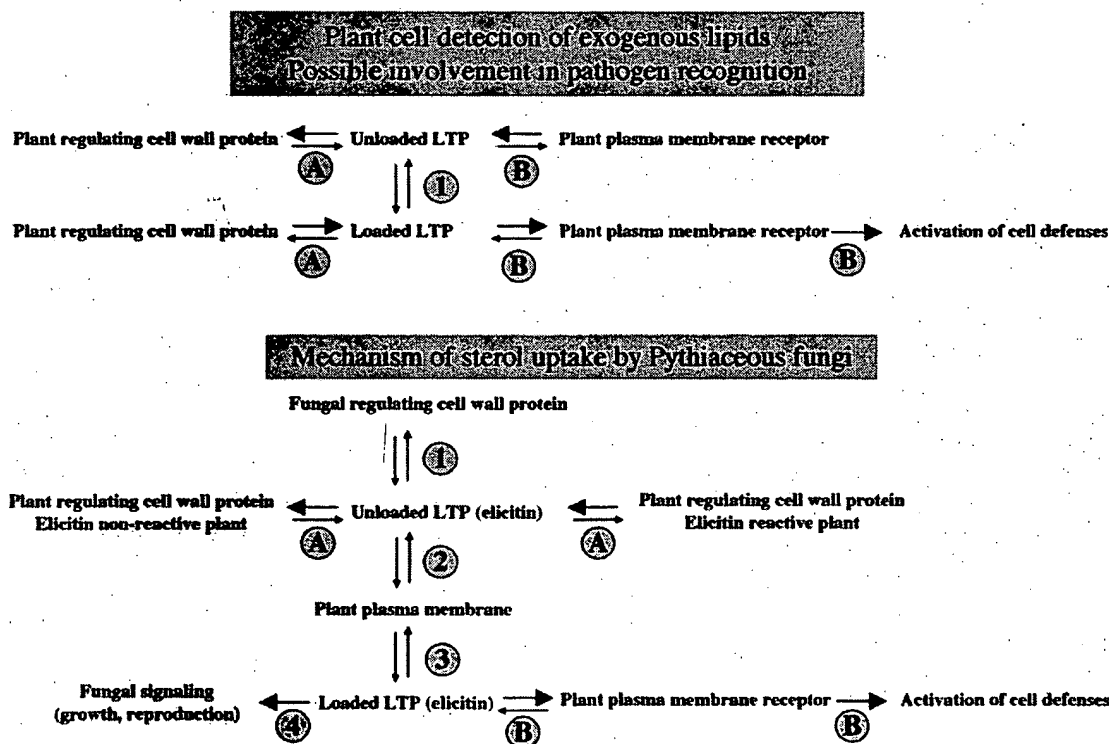

Figure 9. Hypothetical support of plant reactivity/nonreactivity to elicitins. The numbered steps (1–4) represent LTP activation through protein loading with sterols. *A* and *B* are relative to LTP interactions with cell wall-regulating proteins and plasma membrane receptors, respectively. Unloaded LTPs are presumed to exhibit a high or a low affinity for these two types of plant proteins, respectively, in contrast to loaded LTPs.
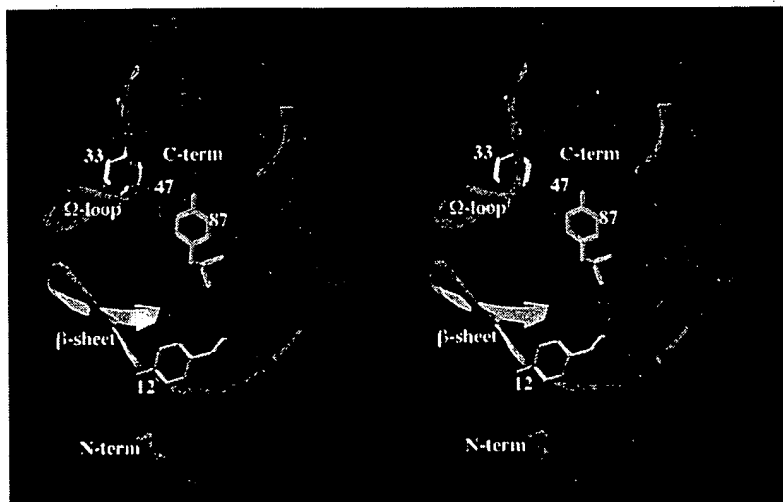
Figure 10. Ribbon diagram of the cryptogein unloaded/loaded with an ergosterol molecule, showing the location of the mutated Tyr residues. It represents the native cryptogein (PDB file 1beo) and the K13H (PDB file 1bxm). Figures were generated using the program Swiss-Pdb Viewer ver. 3.5b1 (http://www.expasy.ch/spdbv/mainpage.htm, and [166, 167]), the image renderer Quick Time ver. 3 (Apple Computer) and the ray tracer POV-Ray ver. 3.1 (http://www.povray.org/).

phobic core was presented [146]. This protein was obtained by overexpression of a synthetic gene encoding cryptogein [112] in the Pichia pastoris heterologous system [147]. The purified and crystallized mutated protein was found to contain a well-defined electron density in its cavity which was identified as ergosterol, the major sterol of yeasts [146]. The presence of a sterol in the mutated cryptogein resulted in slight but important structural changes compared with the native form of the protein previously resolved as crystal [33] and solution [34] structures. These changes concern first some hydrophobic amino acids of the core which were rejected to increase the cavity size, especially Tyr 87, which appears buried in native structure and rotates to be solvent exposed when the sterol is present (fig. 10). Second, a bending of helix α_1 was also reported. Ergosterol seemed to be stabilized in the cryptogein pocket by a hydrogen bond between the phenolic function of Tyr47 and the β-hydroxyl of the sterol, as well as with 28 van der Waals interactions between the sterol rings and side chain and 16 hydrophobic residues of the protein core [146]. These results confirm the biophysical demonstration of the sterol carrier activities of elicitins [59, 83, 84].

Relationship between sterol carrier and biological activities

The link between the two functions of elicitins was assessed using a site-directed mutagenesis strategy. Tyrosine residues (fig. 10), previously suspected to be involved in a protein-ligand complex [146], were methodically replaced, and mutation effects were tested for sterol carrier properties as well as for biological activities on tobacco cells and plants. These mutations resulted in a decrease of all the assayed activities. Moreover, strong correlations could be established between sterol carrier ability and biological functions, and between the rate of elicitins loaded with sterols and their capability to bind specific high-affinity proteins, located on the plasmalemma. These observations can be extended to all the natural elicitins assayed, indicating that the biological activity of these proteins depends on their ability to load sterols [J.-P. Blein et al., unpublished data]. These results indicate that the formation of a sterol-elicitin complex is a requisite step before elicitin binds to high-affinity proteins, which thus constitute their biological receptors. Consequently, this complex formation is the first event involved in elicitin-plant cell interactions [J.-P. Blein et al., unpublished data]. The characteristics of the binding curve kinetics highlighted a cooperative phenomenon during the interaction between elicitins and their putative receptors, which suggests a receptor organization model. First, the elicitin receptor must reflect a multimeric organization (allosteric regulation), in which each monomer could be the 200-kDa complex previously described [128]. The elicitin binding to the receptor triggers an allosteric change of its subunits, probably associated with a phosphorylation event [J.-P. Blein et al., unpublished data]. Second, the calcium signaling in tobacco cells treated

with elicitins shows the following characteristics: (i) protein phosphorylation is required [132, 134], (ii) verapamil and nifedipine, which block voltage-dependent calcium channels in plant cells [148], had no effect on Ca^{2+} influx, indicating that if calcium channels are involved in cryptogein-induced influx, they are not of the voltage-gated type but probably of ligand-dependent type [132], (iii) transient Ca^{2+} uptake can be induced by four sequential elicitin additions [134] and (iv) the mutated cryptogein (Tyr87-Phe) provokes a decrease of the spontaneous Ca^{2+} exchanges in tobacco cells [J.-P. Blein et al., unpublished data]. Taking into account these results, we propose that the elicitin receptor could be a ligand-dependent calcium channel comprising a quadrimeric complex as shown in figure 11, which summarized the initial molecular events involving activation of elicitin by sterol loading that drive elicitor function.

Sterols in oomycete physiology

The dependence toward sterol among the Oomycetes still remains debated. Some of them could synthesize these molecules, and *Achlya ambisexualis*, for example, uses them as precursors of sexual hormones involved in the formation of either oogonia (oogoniol) or anthe-

Figure 11. First events in elicitin signaling. The receptor of elicitins, located on the plant plasma membranes, is presumed to be a calcium channel, comprising four basal subunits (a 160-kDa and a 50-kDa protein), each of them able to specifi-cally bind an elicitin molecule (as shown in fig. 8). The first elicitin-channel interaction needs a loaded elicitin from plant plasmamembrane sterols and triggers a conformational change of the channel, probably associated with the phosphorylation of the subunit bound to elicitin. This conformational modification allows the binding of other loaded/unloaded elicitin molecules to the receptor, and then to trigger biological responses only when this elicitin is loaded.

ridia (antheridiol) [149]. In contrast, numerous Oomycetes belonging to *Pythiaceae* and Lagenidiales are unable to use squalene for the biosynthesis of the steroid nucleus [150]. Thus these microorganisms are completely devoid of sterol equipment. To what extent they truly need these molecules is an open question. For several decades it was considered that the pythiaceous *Pythium* and *Phytophthora* spp. as well as the mosquito parasitizing *Lagenidium giganteum* require sterols for efficient growth and for sexual and asexual reproduction [151–153]. In fact, this is only partially true. It is obvious that sterols provided in artificial growing conditions trigger the formation of reproductive organs in both homo- and heterothallic mycetes. But a lack of sterol supply never affects the fungal growth of *P. cactorum* [M. Ponchet et al., unpublished data]. Stimulation of reproduction organ formation could be obtained by bringing phospholipids to *P. cactorum* [154, 155] or to *Pythium aphanidermatum* [156], even with synthetic compounds [M. Ponchet et al., unpublished data]. This later result excludes that the biological activity of phospholipids results from their contamination by sterols, in contrast to previous conclusions [157]. It was also reported that unsaturated fatty acids as well as their triglycerides were good inducers of reproduction in *P. cinnamomi* [158] and in both *P. cactorum* and *P. parasitica* [159]. In addition, other lipidic compounds such as phytol, a degradation product of chlorophylls, was found to stimulate the reproduction of *P. cactorum*. Concerning the potent structural requirement for sterols in pythiaceous membranes, it was suggested that these compounds could be replaced by triterpenoids [160] such as phytophthorol [161] which are synthesized by these microorganisms and mimic sterol as far as structural and biochemical features are concerned.

In conclusion, sterols constitute powerful signaling components for *Pythiaceae* and Lagenidiales, but are not necessarily required in the physiology of these Oomycetes. According to this conclusion, what is the interest for *Phytophthora* and *Pythium* to secrete high amounts (high energy cost) of different proteins (high genetic diversity) able to transport lipophilic compounds that are not essential for their spreading and dissemination? First of all, this argumentation is built from in vitro observations and cannot prefigure reality during the parasitism of these Oomycetes. For example, the level of elicitin biosynthesis in planta is unknown, even though it was reported that INF1 mRNA was downregulated in potato during the early stages of *P. infestans* colonization [43] and during host pathogen confrontations. Are these proteins free shuttles, as is suggested from biophysical experiments together with abundant secretion in liquid cultures? More probably, these elicitins are sequestered in plant cell walls (fig. 9) or flattened between plant and *Phytophthora* mem-
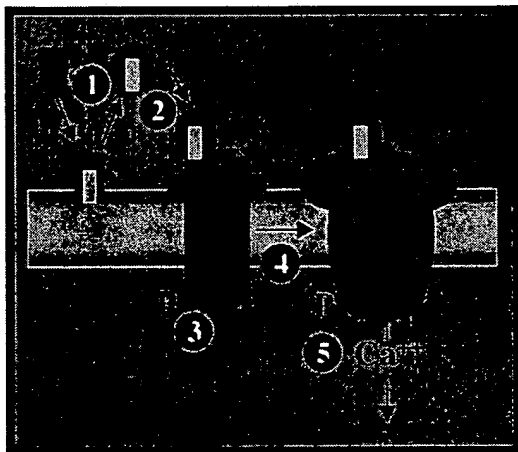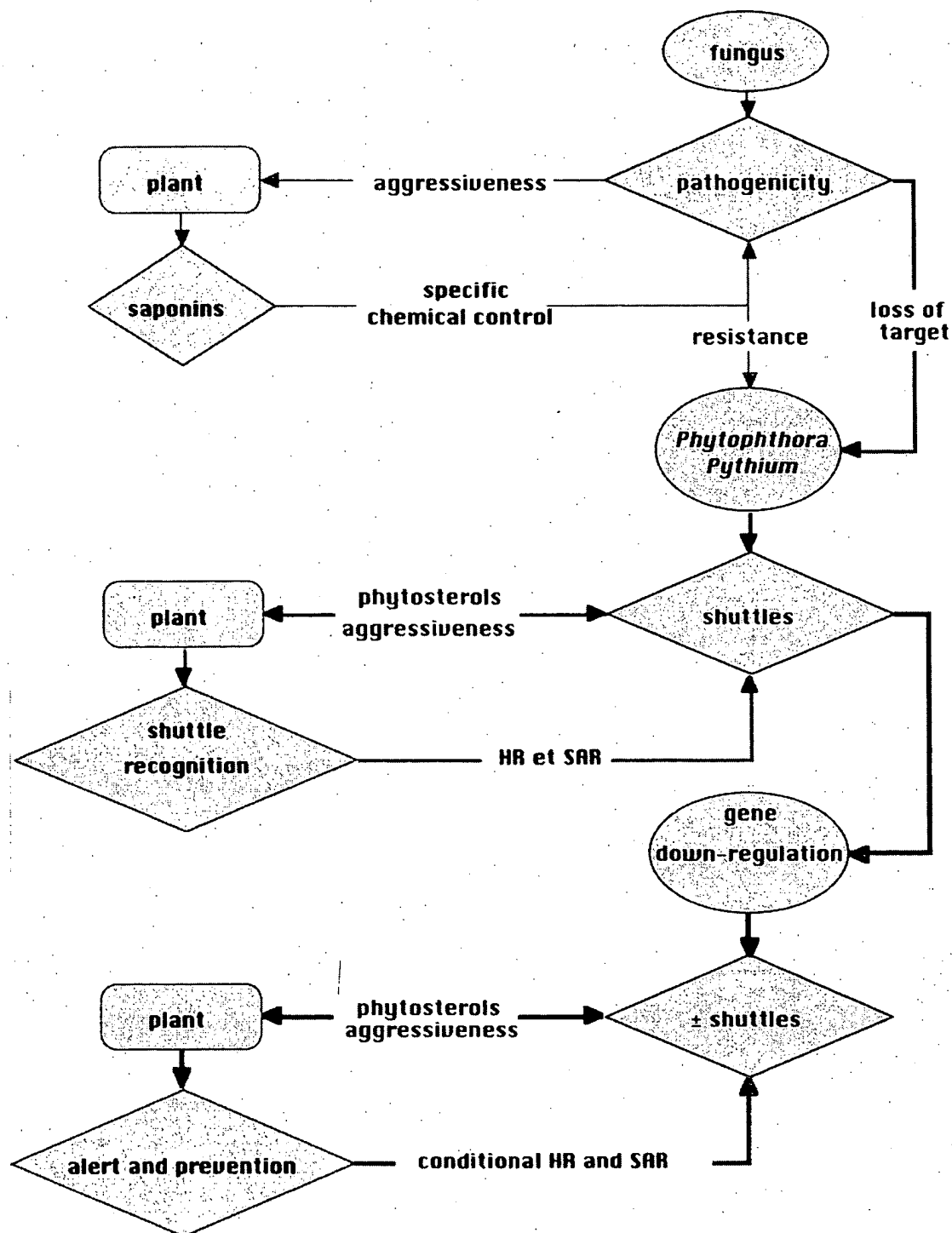
Figure 12. Possible coevolution scheme between plants and *Phytophthora*. Green and pink illustrations are relative to the hypothetical plant and fungal evolutions, respectively.

branes in haustoria or other functionally related structures during plant cell predation. In the latter case, elicitins cannot be viewed anymore as random shuttles. But in every scheme a question remains: why pick up sterols or other lipidic compounds that are not essential from a trophic point of view? An attractive hypothesis is that these proteins are distributed in the oomycete environment to gather foreign lipidic compounds that, by random return to the mycelium, inform the microorganism on the presence and (or) abundance of potential host. Are elicitins sensors for *Phytophthora*? In that way, a more general approach including other interactions, such as the mycoparasitism of *Pythium oligandrum* toward *Fusarium oxysporum* pathogen on tomato [24, 162] is in progress. This particular *Pythium* secretes an elicitin-like protein (oligandrin) able to carry sterols. Thus, this protein was presumed to pick up ergosterol from *F. oxysporum* (involvement in mycoparasitism?); then, during hyperparasitism in planta, oligandrin could interact with the plant system devoted to ergosterol detection [163], as proposed in figure 9.

As a matter of fact, the elicitins analyzed from the sterol point of view appear obviously as components of the virulence of both *Phytophthora* and *Pythium*. Thus the interaction between elicitins and tobacco is the exception in which a general virulence factor is recognized by the host cell and perfectly illustrates host pathogen coevolution.

Recent advances in the knowledge of tobacco/*Phytophthora* interactions reported in this review suggest a hypothetical coevolution scheme of this relationship (fig. 12). Plants have developed several resistance mechanisms, among them the saponin synthesis, which could play an essential role in plant-fungi interactions [164]. These compounds interact with the fungal sterol, and some Oomycetes such as *Phytophthora* and *Pythium* could have short cut this plant aggressiveness by repressing their sterol biosynthesis. Thus they became resistant to saponins and were again able to invade saponin-producing plants. However, they had to pick sterols up from their environment. Then they developed shuttle proteins like elicitins, and the plant invasion could continue. However, although it is now impossible to say which, plant or fungus, has mimicked the other, these elicitins should be homologous with plant proteins involved in in planta signaling, for example, in ergosterol detection, since this fungal sterol induces plant defense mechanisms (fig. 9 [163]). Consequently, after sterol loading, elicitins could be recognized by the warning system of the plant and then trigger a hypersensitive reaction associated with development of SAR. Moreover, although elicitin secretion in planta is poorly documented, downregulation of elicitin production has been reported [43]. Finally, both protagonists still have time to improve their own strategies, and the challenge can continue.

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